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Erratum

In Article 16725 on *Protection of Gastric Mucosa*, Amisate and Protolysate in the tables refer to Hydrolysate in the text.

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Erratum

In Article 16647P, October 1948, p. 150, the Dibenamine used was received in January 1947. On comparison with a newly obtained lot, the older material contained only $\frac{1}{3}$ to $\frac{1}{4}$ its original strength. The dosage therefore stated in the article was too large by the corresponding amount.

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Effect of Feeding Moderate Levels of Commercially Agenized Flour
to Dogs.*

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and Medical Electronics, School of Medicine, University of Wisconsin, Madison.*

During the past 18 months many questions have been raised concerning the role of nitrogen trichloride-treated flour (agenized) in the etiology of human epilepsy. Shortly after Erickson *et al.*¹ suggested that wheat gluten might be important in the causation of human epilepsy, a report by Mellanby² showed that epileptiform convulsions in dogs could be consistently induced when the animals were fed rations which contained high amounts of flour treated with nitrogen trichloride. These observations were later confirmed by Silver *et al.*³ and Newell *et al.*⁴ and the studies were extended to other animals. In all these experiments the rations fed to dogs contained large amounts of flour (70-84%). These

flours were treated with nitrogen trichloride in amounts which ranged up to 15 times more than the treatment normally given flour consumed by human beings.

The use of agene as a maturing agent has increased steadily during the past 25 years. In 1945, 85% of the flour used in this country was treated with nitrogen trichloride. Because of the wide use of wheat products, it was decided to place a series of dogs on a diet which contained an amount of flour approximately equal to that used in the average American diet. This flour was treated with nitrogen trichloride at commercial levels commonly used by the milling industry.[†]

The following ration was used: flour 30, sucrose 49, casein 10, corn oil 5, salts IV 4, whole liver substance 2. Oral supplements of 0.75 mg thiamine, 0.75 mg riboflavin, 0.5 mg pyridoxine, 0.25 mg pantothenic acid, 1 mg nicotinic acid and 70 mg choline per kilo of body weight and 6 drops of halibut liver oil were administered twice weekly. Three dogs, 19, 23 and 30), were placed on ration F-89 which contained flour treated with 1.2 g nitrogen trichloride and 7 g benzoyl peroxide[‡] per cwt. of flour. Two other dogs, (241 and

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¹ Erickson, T. C., Gilson, W. E., Elvehjem, C. A., and Newell, G. W., *Proc. Assn. Research in Nervous and Mental Dis.*, 1946, **26**, 164.

² Mellanby, E., *Brit. Med. J.*, 1946, **2**, 885.

³ Silver, M. L., Johnson, R. E., Kark, R. M., Klein, J. R., Monahan, E. P., and Zevin, S. S., *J. Am. Med. Assn.*, 1947, **135**, 757.

⁴ Newell, G. W., Erickson, T. C., Gilson, W. E., Gershoff, S. N., and Elvehjem, C. A., *J. Am. Med. Assn.*, 1947, **135**, 760.

[†] We are indebted to Dr. H. K. Parker of Wallace and Tiernan Company for the flours used in these experiments.

244), were started on ration F-89A which contained flour treated with 2.25 g of nitrogen trichloride and 7 g of benzoyl peroxide[‡] per cwt. of flour.

Dogs 19 and 23 have been on ration F-89 for 63 weeks (14½ months) and dog 30 has been fed the same ration for 55 weeks (12½ months). Dogs 241 and 244 have been fed ration F-89A which contains 30% flour treated with the higher level of nitrogen trichloride, for 52 weeks (12 months). All of these animals have eaten well, gained weight and remained healthy. At no time has there ever been any indication of running fits or epileptiform convulsions.

Before this experiment was begun characteristic electroencephalographic brain wave patterns (E.E.G.s) of dogs 19, 23 and 30 were obtained.[§] Four tantalum electrodes were implanted into the skull above the dura of each animal in the manner described by

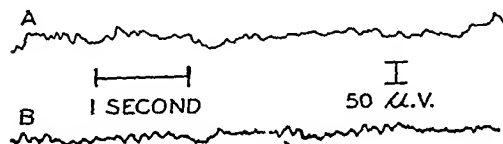


FIG. 1.

A—Control electroencephalogram of dog 30 before experiment.

B—Electroencephalogram of dog 30 after 6½ months on a ration which contained 30% of commercially agenzized flour.

Newell *et al.*⁵ Normal E.E.G. records were obtained for each of these animals. Six to 8 months after these experiments started tantalum electrodes were implanted in all 5 dogs. A week later several E.E.G. records were taken and all the animals showed normal electroencephalograms. In Fig. 1 short sec-

tions of the record from one electrode lead of dog 30, characteristic of the entire record of this dog and of the other dogs, are presented to show the similarity of the E.E.G.s before the feeding tests began and during the period of testing. Previous experiments, in which dogs have received flour or wheat gluten treated with high levels of nitrogen trichloride, have consistently showed abnormal changes in the E.E.G. records days before running fits or epileptiform convulsions developed.⁵ If any abnormalities had developed in the brain metabolism or physiology of the dogs fed rations containing 30% of flour treated with agene at commercial levels, significant changes in the E.E.G. records should have been readily observed.

It is interesting to note here that the flour used in these experiments when fed to dogs at 84% of the ration, regularly caused running fits and convulsions in 11-62 days.⁴ This difference in flour intake suggests that there is a tolerance level in the dog below which the toxic factor produced by nitrogen trichloride treatment is metabolized or excreted but above which the toxic material accumulates in the animal producing the characteristic convulsive condition. When higher levels of nitrogen trichloride are used to treat flour, smaller amounts of flour induce the convulsions. Radomski *et al.*⁶ have recently shown that dogs fed 0.1 g of nitrogen trichloride treated wheat gluten (6 g NCl_3/kg gluten) per kilo of body weight per day did not develop running fits. These authors translate their results to mean that a dog could consume 25% of his ration in the form of commercially treated flour (1.5 g $\text{NCl}_3/\text{cwt.}$) without developing fits.

Summary. These results demonstrate that dogs can be fed a ration containing moderate levels (30%) of commercially agenzized flour for long periods of time (12-14½ months) without developing running fits or electroencephalographic abnormalities.

[‡] Previous tests have shown benzoyl peroxide bleached flour to be ineffective in producing fits.⁴

[§] We wish to express our thanks to Dr. Henry M. Suckle for his assistance in some of the experiments.

⁵ Newell, G. W., Erickson, T. C., Gilson, W. E., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 115.

⁶ Radomski, J. L., Woodward, G., and Johnston, C. D., *Fed. Proc., Abstracts*, 1948, **7**, 250.

Lipotropic Effect of Liver Extract on Dietary Hepatic Injury in Rats.*

CHARLES A. HALL[†] AND VICTOR A. DRILL.[‡]*From the Department of Pharmacology, Yale University School of Medicine, New Haven, Conn.*

Many different supplements have been studied for their effect in preventing liver injury produced by dietary means. The possible effect of liver extract, however, has received little attention. Rhoads and Miller¹ observed that liver extract only partially restored to normal the abnormal liver function produced by a black-tongue diet in dogs. György and Goldblatt² studied the effectiveness of a liver concentrate in preventing dietary cirrhosis in rats and concluded that the "liver extract was completely ineffective in preventing hepatic injury". Gillman and Gillman³ reported that liver extract had some lipotropic effect on the fatty livers of infants with pellagra. In view of the lack of experimental data on the effect of liver extract on experimental dietary liver disease, it was felt that further studies should be made.

Methods. Male rats of the Sprague Dawley strain, weighing between 150 and 170 g were used. All animals received food *ad libitum*. Five animals of each group were housed in individual cages, and their daily food intake was measured. The diet selected was one recently used by Himsworth and Glynn⁴ which is high in fat but not low in protein

and which produced fatty changes followed by fibrosis. The diet consisted of 16% casein, 51% lard, 30% cornstarch, and 3% salt mixture. Vitamins A and D were supplied by adding 5 drops of oleum percomorphum[§] per kilo of diet to the melted lard as the diet was prepared. Each rat received a daily oral supply of the B vitamins as follows: thiamin, 20 µg; riboflavin, 25 µg; pyridoxine, 20 µg; calcium pantothenate, 100 µg. One group of 13 rats received the basic diet alone. The second group of 10 rats received a supplement of 1 cc of Lilly's Crude Liver Extract[†] (1 unit per cc) 3 times a week subcutaneously. A third group of 10 animals received 40 mg of choline chloride daily given subcutaneously in a 4% solution.

After 12 to 13 weeks of the study, a liver biopsy was performed on the 5 animals in each group in the individual cages. The biopsy was carried out by laparotomy under ether anesthesia, and tissue was taken from the edge of the left lobe. At 22 weeks a second biopsy was performed on the same animals in a similar manner. At 29 weeks all surviving animals were killed, and sections were taken for histological examination from the left lobe and the caudate lobe of the liver. The system of grading histological changes is listed with Table I. All liver sections were stained with hematoxylin and eosin. In addition, for half of the animals in each group, frozen sections were made and stained with Sudan III. The remaining liver was analyzed for total lipids by a modification of the

* This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service, and by a grant from Eli Lilly and Company.

[†] Post-War Fellow, Rockefeller Foundation, 1947-1948.

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¹ Rhoads, C. P., and Miller, D. K., *J. Exp. Med.*, 1938, **67**, 463.

² György, P., and Goldblatt, H., *J. Exp. Med.*, 1942, **75**, 355.

³ Gillman, T., and Gillman, J., *Arch. Int. Med.*, 1945, **76**, 63.

⁴ Himsworth, H. P., and Glynn, L. E., *Clin. Science*, 1944, **5**, 93.

[§] The authors wish to thank Dr. K. K. Chen of Eli Lilly and Company for the Liver Extract and Dr. C. E. Bills of Mead Johnson and Company for the Oleum Percomorphum. Each cubic centimeter of liver extract contained: Thiamin, 2.0-4.5 µg; Riboflavin, 45.0-60.0 µg; Pantothenic acid, 45.0-60.0 µg; Nicotinic acid, 100.0-140.0 µg; Folic acid, 2.0-3.8 µg; Methionine, 3.0 µg; and Choline, 1.0-1.6 µg. Total solids, 16-18%.

244), were started on ration F-89A which contained flour treated with 2.25 g of nitrogen trichloride and 7 g of benzoyl peroxide† per cwt. of flour.

Dogs 19 and 23 have been on ration F-89 for 63 weeks (14½ months) and dog 30 has been fed the same ration for 55 weeks (12½ months). Dogs 241 and 244 have been fed ration F-89A which contains 30% flour treated with the higher level of nitrogen trichloride, for 52 weeks (12 months). All of these animals have eaten well, gained weight and remained healthy. At no time has there ever been any indication of running fits or epileptiform convulsions.

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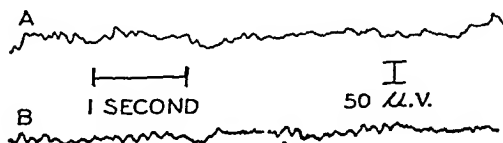


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⁵ Newell, G. W., Erickson, T. C., Gilson, W. E., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 115.

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It is interesting to note here that the flour used in these experiments when fed to dogs at 84% of the ration, regularly caused running fits and convulsions in 11-62 days.⁴ This difference in flour intake suggests that there is a tolerance level in the dog below which the toxic factor produced by nitrogen trichloride treatment is metabolized or excreted but above which the toxic material accumulates in the animal producing the characteristic convulsive condition. When higher levels of nitrogen trichloride are used to treat flour, smaller amounts of flour induce the convulsions. Radomski *et al.*⁶ have recently shown that dogs fed 0.1 g of nitrogen trichloride treated wheat gluten (6 g NCl_3/kg gluten) per kilo of body weight per day did not develop running fits. These authors translate their results to mean that a dog could consume 25% of his ration in the form of commercially treated flour (1.5 g $\text{NCl}_3/\text{cwt.}$) without developing fits.

Summary. These results demonstrate that dogs can be fed a ration containing moderate levels (30%) of commercially agenzized flour for long periods of time (12-14½ months) without developing running fits or electroencephalographic abnormalities.

⁶ Radomski, J. L., Woodard, G., and Johnston, C. D., *Fed. Proc., Abstracts*, 1948, **7**, 250.

method of Outhouse and Forbes.⁵

Results. The untreated animals fed the high-fat diet showed a marked increase in liver fat histologically, whereas treatment with liver extract or choline prevented the fatty changes in the liver (Table I). As indicated in Table I several animals died prior to the 29th week, generally from liver injury associated with infection.

In the untreated animals the change observed at the first biopsy was a marked infiltration with large fat droplets which almost completely filled the hepatic cells, pushing the nucleus and remaining cytoplasm into a narrow rim. The end result seen in the autopsy sections was a trabeculation of the entire liver with strands of fibrous tissue dividing the liver into sections smaller than the normal lobule. Large, isolated, fat-free hepatic cells with heavily staining cytoplasm and one or two large, dark staining nuclei were occasionally seen enmeshed in the fibrous strands. The progressive nature of the fibrosis in the untreated animals may be observed in the data in Table I. The fibrosis was never present unless there was also a marked fatty change. In following those animals with marked fatty change and fibrosis through biopsies to autopsy it was observed that as the fibrosis increased there was a slight decrease in the amount of fat in the cells.

The animals treated with liver extract or choline did not show any fatty change in the left lobe of the liver at the first or second biopsy or at autopsy. However, in a few of the animals in these groups additional examination of the caudate lobe at necropsy showed a slight (1+) fatty change (Table I). However, this is a normal variation between lobes of the liver as other animals fed a normal diet of 16% casein, 6% fat, 75% corn starch, and 3% salt mixture showed a similar variation between left and caudate lobes in some cases.

The results of the lipid determinations are shown in Table II. The untreated animals showed a definite increase in lipid concentra-

TABLE II.
Total Liver Lipids in % of Wet Weight.

Basic Diet		Liver Extract		Choline	
Rat No.	% Fat	Rat No.	% Fat	Rat No.	% Fat
1	27.3	14	8.5	24	8.9
2	25.8	15	10.4	25	6.2
3	18.2	16	7.5	26	7.6
4	27.1	17	6.4	27	7.4
5	20.1	18	7.1	28	8.3
6	14.3	20	11.9	30	8.3
8	20.4	21	9.2	31	10.2
12	15.1	22	11.2	32	5.4
13	15.9	23	7.4	33	3.9
Mean	20.50		8.84		7.36
S.E.	±1.72		±.65		±.64

tion. Treatment with liver extract or choline prevented the increase in liver lipids, and the resulting concentration of lipids in these animals are in the same range as rats fed a normal low-fat diet (16% casein-6% fat). Lipid determinations were not performed on the animals that died early in the experiment or who showed postmortem changes.

Discussion. Previous investigators have produced similar hepatic lesions of fatty change and fibrosis in rats by dietary means.^{4,6} Others have produced a dietary injury consisting of fatty change, fibrosis, and necrosis.^{2,4,7,8} Necrosis was not observed in any of the animals in the present study. All of the diets that have produced necrosis differ from that used here in several aspects. An important difference that is usually associated with the occurrence of necrosis is the use of diets which are low in protein, generally ranging from 6% to 10% protein. In the present study the diet contained 16% protein.

The supplement of choline prevented both the infiltration of the liver with fat and the fibrosis which followed the fatty change in untreated animals. This lipotropic effect of choline is well known, but a lipotropic effect of liver extract as obtained in the present study, has not been reported previously.

György and Goldblatt² studied the effect of daily doses of 0.2 g of a liver concentrate

⁶ Blumberg, H., and Grady, H. G., *Arch. Path.*, 1942, **34**, 1035.

⁷ Handler, P., and Dubin, I. N., *J. Nutrition*, 1946, **31**, 141.

⁸ Webster, G. T., *J. Clin. Invest.*, 1942, **21**, 385.

⁵ Outhouse, E. L., and Forbes, J. C., *J. Lab. and Clin. Med.*, 1940, **25**, 1157.

TABLE I.
Histological Changes in Liver at Biopsy and Necropsy.

Rat No.	Basic diet				Liver extract				Choline			
	Wt change	Fat		Fibrosis	Rat No.	Wt change	Fat		Rat No.	Wt change	Fibrosis	
		L.L.	C.L.				L.L.	C.L.			L.L.	C.L.
1	+66	4+		0	14	+66	0		24	+175	0	
2	+56	4+		0	15	+99	0		25	+126	0	0
3	+55	3+		1+	16	+90	0		26	+110	0	0
4	+74	3+		0	17	+100	0		27	+71	0	0
5	+32	3+		0	18	+124	0		28	+112	0	0
1	+80	4+		1+	14	+81	0		24	+179	0	0
2	+58	4+		1+	15	+107	0		25	+159	0	0
3	+60	4+		2+	16	+106	0		26	+116	0	0
4	+67	3+		0	17	+111	0		27	+71	0	0
5	+23	3+		0	18	+127	0		28	+112	0	0
1	+58	4+		2+	14	+48	0		24	+42	0	0
2	+28	3+		2+	15	+65	0		25	+50	0	0
3	-16	4+		2+	16	+52	0		26	+52	0	0
4	+30	4+		2+	17	+50	0		27	+65	0	0
5	+3	4+		0	18	+68	0		28	+35	0	0
6	+56	3+		1+	19	+57	0		29	+59	0	0
7*	-32	4+		2+	20	+82	0		30	+88	0	0
8*	+22	4+		2+	21	+86	0		31	+108	0	0
9*	+3	4+		1+	22	+98	0		32*	+86	0	0
10*	+26	4+		0	23	+28	0		33*	+52	0	+
11*	+40	4+		2+								
12*	+10	4+		2+								
13*		4+		2+								

*Comments.

No. 7 died at 148 days of pneumonia.

No. 8 " " 107 " from anaesthesia.

No. 9 " " 67 " no infection found.

No. 10 " " 70 " " "

No. 11 " " 72 " " "

No. 12 died at 165 days of pericarditis.

No. 13 " " 170 " " "

No. 32 " " 173 " " pneumonia.

No. 33 " " 186 " " "

Grading of Fatty Change.

0—None or only a rare large droplet.

++—A few large droplets in each lobule.

+++—More than ++, but less than half of lobule involved.

++++—Over half of lobule involved but some cells fat free.

++++—Almost no fat free cells.

L.L. = Left lobe of liver; C.L. = Caudate lobe of liver.

Grading of Fibrosis.

0—None.

++—Thin strands of fibrous tissue scattered throughout.

+++—Strands of fibrous tissue dividing liver into small sections.

Failure of Splenectomized Rabbits to Respond to Liver Extract and to Pteroylglutamic Acid.*

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Jacobson and Williams^{1,2} reported that splenectomized rabbits responded to the administration of several materials with an increase in reticulocytes. Liver extracts effective in the treatment of pernicious anemia, synthetic xanthopterin, leucopterin and the pigment derived from fused uric acid caused reticulocytosis in their animals. Clinically inactive liver extracts as well as a number of unrelated materials failed to evoke a response. Substances inducing reticulocyte increases were effective when given either intramuscularly, intravenously or orally. Based on a large number of control counts it was concluded that any reticulocyte value exceeding 3.5% following administration of a trial material indicated hemopoietic activity of that material. It is to be noted that none of their animals was anemic at any time during the period of observation.

The nature of the materials found effective by these authors stimulated us to test in a similar fashion the newly synthesized pteroylglutamic acid (PGA)³ which had been found to be effective in the treatment of the macrocytic anemia of sprue⁴ and in pernicious anemia.⁵

Procedure. Nine adult white rabbits were used. The animals were fed Purina rabbit chow exclusively throughout the period of experimentation. Eight were males. The spleen was removed from 6 of these animals; the remaining 3 served as normal controls. Of the 6 splenectomized rabbits 4 were treated and 2 were followed as splenectomized controls. There was very little loss of blood from operation. However, to avoid reticulocyte increases owing to blood loss, 3 weeks were allowed to pass following operation before any trial material was given. Pre-operative and post-operative red blood cell counts and determinations of hemoglobin indicated no anemia by standards reported for rabbits.⁶

Rabbits No. 1 and 2 (splenectomized) were given 5 mg of PGA intramuscularly on 4 occasions over a period of 8 days. Rabbits No. 3 and 10 (splenectomized) were given 5 units of liver extract (Wilson) intramuscularly on 3 occasions over a period of 7 days. No reticulocyte increases having occurred by 10 days following the last injection of a series the animals were re-treated after a rest period of 10 days. Animals No. 1 and 2 were given 20 mg of PGA intramuscularly on 3 occasions within a 5 day period. Rabbits No. 3 and 10 were given 20 units of liver extract (Wilson) intramuscularly on 3 occasions over a period of 5 days. No reticulocyte responses having followed, liver extract and PGA were then given into the stomach by means of a tube. Animals No. 1 and 3 were given 50 units of liver extract (Wilson) by tube on 3 occasions over a period of 7 days. Animals Nos. 2 and 10 were given 10 mg of PGA by tube on 3 occasions over a 7 day period.

* This work was supported by a grant from The National Vitamin Foundation.

¹ Jacobson, W., and Williams, S. M., *J. Path. Bact.*, 1945, **57**, 101.

² *Ibid.*, 1945, **57**, 423.

³ Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., Subbarow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Siekels, J. P., and Smith, J. M., Jr., *Science*, 1945, **102**, 227.

⁴ Darby, W. J., and Jones, E., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 259.

⁵ Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D., *J. Lab. and Clin. Med.*, 1945, **30**, 1056.

⁶ Wintrobe, M. M., Shumacker, H. B., Jr., and Schmidt, W. J., *Am. J. Physiol.*, 1936, **114**, 502.

TABLE III.
Average Daily Intakes of Food, Protein, and Calories.

Treatment	Food g/rat	Protein g/rat	Protein G per 100 g rat	Calories per rat	Calories per 100 g rat
Basic diet	7.2	1.15	0.56	46.3	22.4
Liver extr.	8.0	1.28	0.54	51.6	21.6
Choline	9.1	1.45	0.55	58.5	22.1

in preventing the hepatic injury of fatty change, cirrhosis and necrosis produced in rats by a low-protein, high-fat diet. They concluded that the liver extract failed to prevent the induced liver injury. Either the differences in the types of injury produced, or in the liver concentrate used by György and Goldblatt may be responsible for the lack of effect of their concentrate as compared with the beneficial effect of liver extract in the present study. The animals in the present study received on the basis of total solids the equivalent of 0.51 g of powdered liver No. 343 per rat per week as compared to the 1.4 g of concentrate used by György and Goldblatt.

The effect of the liver extract does not appear to be due to an increase in protein intake resulting from stimulation of the appetite by the liver. The protein intake per rat was only slightly higher in the liver-treated group as compared to the untreated group, and the protein intake per 100 g of rat weight was the same in both groups (Table III). A group of animals not included in this report were given the basic diet with an additional 8% casein. This resulted in an average protein intake per rat of 2.12 g per day, and a protein intake of 0.79 g per 100 g of rat weight per day. Even with this protein intake, which is considerably above that of the liver extract and choline treated animals, all of these animals showed a 1+ or 2+ infiltration with fat.

The choline content of the liver extract is not responsible for its effectiveness. The liver extract used contained 1.0-1.6 mg of choline per cubic centimeter, and therefore

each rat received only 0.56 mg of choline a day. Other data in the literature show that larger amounts of choline are necessary to prevent dietary lesions in the liver. The lowest effective dose used was that of Daft, Sebrell and Lillie⁹ who found that a dietary supplement of 20 mg of choline protected against the lesion produced by a 4% protein-5% fat diet. Himsworth and Glynn¹ found that the fat content of the liver was normal at 34 days, if 4 mg of choline daily was used as a supplement to a diet containing 8% protein and 50% fat; but abnormal amounts of fat were present at 66 days in spite of continued choline administration. Furthermore, other supplements which will be reported in detail later, and which failed to prevent the fatty change, had a higher content of choline than the liver extract. It is possible that the liver extract contains an unknown lipotropic substance.

Summary. 1. Rats fed a 16% casein, 51% fat diet for 29 weeks all developed markedly fatty livers. Nearly all of the animals showed a diffuse, progressive hepatic fibrosis.

2. The fatty change and fibrosis were completely prevented by supplementing the high-fat diet with choline, or with crude liver extract.

3. The lipotropic effect of the liver extract is not due to an increase in protein intake resulting from a stimulation of appetite, and is not due to the small amount of choline present in the liver extract.

⁹ Daft, F. S., Sebrell, W. H., and Lillie, R. D., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 228.

tered with anti-M agglutinins produced apparently as a result of transfusions of type M blood into type N individuals. Wiener and Forer³ reported a case in which a woman had hemolytic reactions following blood transfusions, and difficulty was encountered in finding a completely compatible blood in cross-matching tests. On testing the blood of the patient, these investigators found her blood to belong to group O, type N, Rh-negative, while her serum contained anti-M and anti-Rh agglutinins. Broman⁴ has reported a hemolytic transfusion reaction caused by M sensitization.

In analyzing our cases of Rh sensitization, we found that the spacing of injections was far more important in bringing about isosensitization than the quantity of antigen introduced into the body.⁵ In fact, we have applied this principle successfully in devising a simple method of producing anti-Rh serum in normal male type rh donors.⁶ While these investigations on the preparation of anti-Rh sera were being carried out, some observations were made on the isosensitization of normal male individuals to the M agglutino-gen. The purpose of this paper is to describe these observations.

Case I. This group AB, type rh volunteer was given repeated intravenous injections of 2 cc each of whole Rh-positive blood diluted to 5 cc with sodium citrate solution. The first (priming) injection was given in October 1946. The second injection, or first "stimulating" injection, was given in the middle of February 1947. Ten days later the donor's serum was found to contain univalent Rh₀ antibodies (Rh₀ glutinins) of 50 units titer by the plasma conglutination method, together with weak anti-rh" agglutinins. Since the titer was not high enough to yield a usable serum, the donor was not bled at that time, and a second stimulating injection was given

2 months later. After this injection the Rh₀ glutinin titer was found to be 85 units by the plasma conglutination method, while the anti-rh" agglutinin titer rose to 20 units. At this time, we also detected in the volunteer's serum a weak agglutinin reacting with Rh-negative blood but not with the donor's own blood cells. The donor was given a fourth injection of 2 months later, after which the anti-Rh₀ glutinin titer was 512 units by the albumin-plasma method, while the anti-rh" agglutinin titer proved to be 40 units. Again the presence of a third antibody reacting with type rh blood was noted, and it was decided to investigate the nature of the third antibody.

Titration showed the third antibody to be an agglutinin of 3 units titer, approximately equally active at body, room and refrigerator temperatures. Tests were set up against a series of 28 blood samples of type rh and type Rh₁, but otherwise unselected, by the agglutination method at room temperature.* Of the 28 bloods tested, only 2 gave negative reactions, besides the volunteer's own blood. One of the negatively reacting blood samples belonged to type ONRh₁rh and the other to type BNrh. Since these 2 blood samples were the only ones in the series besides the volunteer's which belonged to type N, this suggested that we were dealing with an anti-M immune agglutinin. In fact, further tests proved that all type N bloods of types Rh₁ and rh were not agglutinated by the serum, while all other bloods were agglutinated, confirming the diagnosis of anti-M as the specificity of the antibody.

For subsequent stimulating injections only type N, Rh-positive blood was used, and as shown in Table I, later bleedings obtained from this volunteer contained no demonstrable M antibodies.

Case II. The second volunteer, also group AB and type rh, has not been studied as intensively as the first one. Unlike the first volunteer, this individual produced a potent

³ Wiener, A. S., and Forer, S., *Proc. Soc. Exp. Biol. and Med.*, 1941, 47, 215.

⁴ Broman, B., *Acta Paediat.*, 1944, 31, supp. 2.

⁵ Unger, L. J., and Wiener, A. S., *Am. J. Clin. Path.*, 1945, 15, 280.

⁶ Wiener, A. S., and Sonn-Gordon, E. B., *Am. J. Clin. Path.*, 1947, 17, 67.

* The Rh₀ glutinin in the serum did not clump type Rh₁ bloods under these conditions. Bloods of types Rh₂ and Rh₁Rh₂ could not be used in these tests because of the anti-rh" agglutinin in the serum.

With few exceptions daily reticulocyte counts were made on the 4 splenectomized animals throughout the entire periods following the first treatment. These periods covered approximately 7 weeks. Splenectomized and normal controls were followed with practically daily reticulocyte counts over a period of 3 weeks from the beginning of the experiment. The wet technic for counting reticulocytes was employed using brilliant cresyl blue dye. One thousand cells were counted on each determination. The same 3 observers made all counts. The liver extract used was known to be potent in the treatment of pernicious anemia.

Results. No significant increase in reticulocytes was noted in any of the animals which has been splenectomized and adequately treated with either liver extract or PGA. The normal controls showed reticulocyte ranges from .6 to 5.3%; the splenectomized controls showed ranges of 1.3 to 4.8%; the animals treated showed ranges of .7 to 5.2%, the values bearing no relation to material administered. Chart 1 shows the reticulocyte range of a normal control animal. Chart 2 shows the range of a splenectomized control. No charts are shown for rabbits treated since the reticulocyte ranges of these animals differed in no respects from those shown. The reticulocyte ranges given were experienced

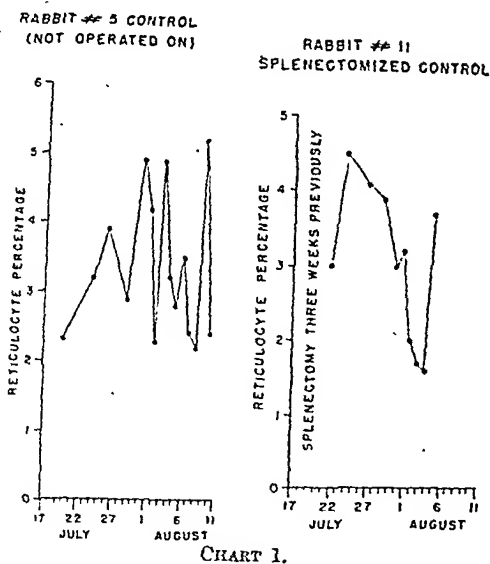


CHART 1.

by practically all of the animals used, regardless of group. No trend in any direction was noted in the case of a single animal.

Conclusions. Splenectomized white rabbits showed no significant change in percentage of reticulocytes following oral and parenteral administration of either pteroylglutamic acid or of liver extract effective in pernicious anemia. Greater variations were found in daily reticulocyte counts, both in controls and in splenectomized animals, than were reported by Jacobson and Williams.

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Observations on Isosensitization to the M Agglutinin in Man.

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It is generally considered that the agglutinogens M and N can be disregarded when selecting donors for blood transfusion therapy. While this is true for patients requiring only a single transfusion, or multiple transfusions within a short space of time, in cases where several transfusions spaced over a long period of time (months or years) are required, one must take into account the possibility of isosensitization to the M agglutinin as well

as the Rh factor.

Only a relatively small number of cases have been reported of type N individuals with natural anti-M agglutinins in their sera.^{1,2} In addition, a few cases have been encoun-

¹ Wiener, A. S., *Blood Groups and Transfusion*, 3rd ed., p. 219, C. C. Thomas, Springfield, Ill., 1945.

² Unger, L. J., Wiener, A. S., and Sonn, E. B., *Am. J. Clin. Path.*, 1946, 16, 45.

TABLE II.
Analogies Among the A-B-O, M-N, and Rh-Hr Systems of Blood Factors.*

System*	Major agglutinogens (More antigenic)	Minor agglutinogens (Less antigenic)
A-B-O	A, B, A ₁ , C†	O(A ₂)‡
M-N	M	N
Rh-Hr	Rh ₀ , rh', rh''	Hr ₀ , hr', hr''

* For simplicity the rare agglutinogens A₃, N₂, rh''', etc., are not included in this table.

† The factor C is shared by bloods containing agglutinogens A₁, B, or both.

‡ The factor O(A₂) is shared by agglutinogens O and A₂, but absent from agglutinogens A₁ and B.

TABLE III.
Allelic Relationships Among the Landsteiner Blood Groups and Subgroups.

C-O genotypes	Reaction with serums		Blood groups	
	Anti-A ₁ B (Anti-C)	Anti-A ₂ O (Anti-O)	Phenotypes	Genotypes
CC	+	—	A ₁ , B, A ₁ B	A ₁ A ₁ , BB, and A ₁ B
CO	+	+	A ₁ , B, and A ₂ B	A ₁ O, A ₁ A ₂ , BO, and A ₂ B
OO	—	+	O, A ₂	OO, A ₂ A ₂ , and A ₂ O

DeKromme and Van der Spek¹⁰ also seems to be an example of natural anti-N rather immune anti-N, as claimed by these workers, considering that their abnormal antibody had the properties of a cold agglutinin.

As is well known, in the Rh-Hr system, the Rh and Hr agglutinogens are related to one another serologically and genetically like the M and N agglutinogens.¹ Of the Rh-Hr agglutinogens, the Rh factors are the more potent antigens and thus correspond to the agglutinogens M in the M-N system, while the Hr factors are the less potent antigens and correspond to the agglutinin N (cf. table II). That similar relationships also exist in

the A-B-O groups is demonstrated in Table II and Table III.

Summary. Two cases are described in which type N individuals produced anti-M agglutinins following injections at widely spaced intervals of blood containing the M agglutinin. On the basis of these and other observations, it is pointed out that type N patients requiring a series of transfusions at wide intervals should be transfused only with type N blood, if isosensitization and consequent transfusion reactions are to be avoided. Genetic and serologic analogies among the A-B-O, M-N and Rh-Hr systems of blood factors are pointed out. Evidence is cited to show that the factors in each system fall naturally into two major subdivisions.

¹⁰ DeKromme, L., and Van der Spek, L. A. M., *Brit. Med. J.*, 1948, 1, 643.

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Cure of Ulcerative Cecitis of Rats by Streptomycin.

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Ulcerative cecitis of rats is a disease of considerable importance from two standpoints. First of all, when prevalent in a

rat colony, experimental work carried out with the affected animals becomes confused, and incorrect conclusions may be drawn.

TABLE I. Results of Immunization Experiment on Two Type N, Rh-Negative Individuals.

Case history	Date*	Agglutinins (titration in saline media)	Rh ₀ antibodies (titer in units†)			Anti-ph ⁺ agglutinins (titration in saline media)	Anti-M agglutinins (titration in saline media‡)
			Blocking test	Univalent antibodies as determined by — Conglutination test in —			
				Plasma	Albumin-Plasma mixture		
Volunteer No. 1, A ₁ BNrh ⁺ Blood used for injection OMRh ₀ Rh ₀	2/26/47	0		50		5	0
	3/ 5/47	0	4	85		2	0
	4/29/47	0	4	85		20	2
	6/18/47	0	4	104	214	16	3
	6/25/47	0	6		512	40	3
	8/27/47	0	12		500	12	0
	12/ 5/47	0			300	40	0
	1/30/48	0	8			160	0
	2/ 9/48	0			192	64	0
Volunteer No. 2, A ₂ BNrh ⁺ Blood used for injection OMRh ₀	8/28/47	640				0	0
	3/ 1/48	704				192	0

* These tests were usually made 10 days after a stimulating injection.

† The number of units equals the reciprocal of the highest serum dilution giving a one-plus reaction.

‡ Clumping weak throughout. § Tests for anti-M done at refrigerator, room, and body temperatures (no difference in titer).

anti-Rh₀ agglutinin of 640 units after the first stimulating injection. Following a second stimulating injection about 6 months later, the Rh₀ agglutinin titer was 70 units but there were also Rh₀ glutinins of a titer of 192 units by the albumin-plasma method, as well as an additional agglutinin of 8 units titer which reacted with type rh blood cells. Specificity tests proved the third antibody to be an anti-M immune agglutinin.

Comment. The observations just described demonstrate the necessity of administering only type N blood to type N patients requiring a series of transfusions at widely spaced intervals. When injections are spaced at sufficiently wide intervals, the agglutinin M seems to be a fairly good antigen for individuals of type N. This was well illustrated by a case concerning which we were consulted several years ago, because of difficulty encountered in the cross-matching tests. This we found to be due to an anti-M immune agglutinin which was active in dilutions as high as 1:256 at body temperature. On the other hand, the N agglutinin appears to be a weak antigen for individuals to type M. Only a single convincing case of isosensitization to the N agglutinin has been reported⁷ in the literature and this involved a patient with an extraordinary capacity to produce antibodies, who in response to a series of transfusions formed in succession antibodies specific for factor hr', N, and 3 previously undescribed agglutinogens.

With regard to the previous report by Singer⁸ claiming isosensitization to the N agglutinin by transfusion, only a few bloods were tested with the patient's serum and this is not adequate to establish the specificity of an abnormal antibody. Natural anti-N agglutinins were found by the late G. L. Taylor in one case (cited by Callender and Race⁹) and another such example was reported by Allott and Holman.⁹ The case reported by

⁷ Callender, A. T., and Race, R. R., *Ann. Eng.*, 1946, 13, 102.

⁸ Singer, E., *Med. J. Aust.*, 1943, 2, 29.

⁹ Allott, E. N., and Holman, C. A., *Lancet*, 1947, 2, 130.

TABLE II.
Cecal Lesions Before and After Streptomycin Therapy.

Rat No.	Appearance at laparotomy May 11	Appearance at autopsy May 20 after streptomycin for 9 days
75275	Mass 1.5 cm with large glands and adherent omentum	Cecum slightly thickened. No open ulcer in cecum. A little thickening of tissues at hilum. Mass is gone.
75213	Mass 0.5 cm. Small adherent glands.	A tiny hard gland at hilum. Mucosa seems a little thickened. No ulcer
7400	Cecum itself is soft, but there is an adjacent mass of glands and cysts 1.5 cm in diameter	Small cecum with adhesions and tiny remains of a gland. Mucosa slightly thickened—no ulcer
74039	Mass 1.5 cm with large glands and cyst 0.5 cm. Much matting	A small mass of firm white glands in hilum. Adhesions to omentum. Mucosa seems thickened but no ulcer
74258	A 2 cm adherent mass of cysts and glands	Mass gone. Adhesions. Mucosa seems slightly thickened. No ulcer
75536	1.5 cm mass of glands and cysts	Cecum practically normal but thickening and adhesions at hilum with a small cyst containing fluid
75775	1.5 cm mass with glands and cysts. Omentum adherent	Mucosa normal. Some scarring and adhesions outside with small hard hilar nodes
76191	Great pericecal adhesions with cyst and small mass in cecum	Cecum scarred and adherent. A large cyst containing fluid. Mucosa slightly thickened—no ulcer
76199	Mass 0.5 cm with adherent omentum and glands	Pericecal adhesions. Mucosa normal—no ulcer
77435	Huge mass nearly 3 cm in diameter. Everything matted and adherent	Remarkable resolution. Many adhesions but no glands. Mucosa much thickened. A gray area nearly 1 cm in diameter marks remains of what must have been a large encrusted ulcer before treatment
76498	0.5 cm adherent mass of glands and cysts	No <i>Streptomycin</i> . Thickened adherent cecum. Huge 2x2 cm typical green ulcer in usual situation in cecum.

130 days old one-half the group were sacrificed and the incidence of cecitis was noted. All but one of the group had definite lesions. One might expect, therefore, a similar incidence of cecitis in the remaining 10 rats. On April 12th streptomycin was dissolved in the drinking water of these animals so that each rat took on the average of 0.1 g of the antibiotic daily. The rats thrive on the streptomycin and soon disdained plain water. During the 10 days of streptomycin therapy all the animals gained weight, some as much as 20 to 25 g. On April 22nd the rats were killed. In contrast to the controls sacrificed 10 days previously none of the treated animals had definite lesions with the exception of one rat which showed the faint remains of a shallow ulcer 0.5 cm in diameter and obviously healing. In rat 12 the cecum appeared normal but a slight glandular enlargement still remained at the hilum. Cultures from the cecums of five rats yielded no growth, and sections through the entire organ showed no active ulcerations except in one case in which there was a small acute shallow ulcer with complete erosion of the mucosa. How-

ever all the cecums showed infiltration of the submucosa with eosinophiles and to a lesser extent neutrophiles, evidently the remains of a healing process.

Streptomycin appears then to have a striking therapeutic effect in ulcerative cecitis of rats. However, it seemed necessary to control our observations even more precisely. The following experiment was therefore carried out:

Experiment II. A number of rats in which a clinical diagnosis of cecitis could be made on the basis of a palpable abdominal mass were collected. On April 11th, exploratory laparotomy was done on 11 animals under ether anesthesia and the condition of the cecum was carefully noted (see Table II). The incisions were closed and streptomycin was immediately added to the drinking water of 10 rats so that each animal took on the average 0.1 g daily. The remaining animal received no antibiotic. After 9 days all the animals were sacrificed. The results are shown in Table II. As in Experiment I a remarkable therapeutic effect is observed. While there were some residues of infection in the way

Secondly, the disease itself is of great interest. Since it is exquisitely chronic and interferes very little with the general health of the animals one has an excellent tool for the study of experimental epidemiology, the pathogenesis of infection and the effects of chemotherapy and antibiotics. Certain aspects of these questions have been dealt with in previous papers.¹⁻⁶

In 1940-41 cecitis became prevalent in the Stanford rat colony. Very young animals were not affected, but by 4-5 months 44% of the stock had fresh lesions and the incidence increased thereafter so that 66% of 7-8 months rats showed lesions. The etiology of this interesting disease is not yet entirely clear. Cultures from the ulcerations have invariably yielded *Salmonella enteritidis*, but in spite of this we have raised the question of whether this organism alone is responsible. If bacteria of the *Salmonella* group freshly isolated from rats with cecitis are introduced into the drinking water of unaffected animals one produces a violent diffuse enteritis which in no way resembles the spontaneous disease. Rats which survive such an acute infection then proceed gradually to develop the usual lesions but with no greater frequency and to no greater extent than uninoculated controls.³ Organisms, furthermore, have not been obtained from fluid aspirated from the cysts which so often develop around the cecum. It was also shown that the development of cecitis was inhibited in rats on a vitamin B deficient diet, a finding just the reverse of what one would expect with an ordinary bacterial infection.⁶

Be all this as it may, we found that cecitis could be prevented by incorporating 1% of succinyl sulfathiazole in the stock diet.² Sul-

faguanidine 0.5% was equally effective.¹ The interesting observation was also made that if sulfaguanidine was added to the diet of the mothers during pregnancy and lactation the young subsequently failed to develop cecitis.⁵ By these means the disease was eliminated from the colony and for the past three years no chemo-prophylaxis has been used.⁴

In the fall of 1947 there were indications that cecitis was again present in the colony, and the incidence was found to be as shown in Table I. The situation was even worse

TABLE I.
Incidence of Cecitis in the Stanford Rat Colony, 1948.

Age of rats (days)	60	70-75	130-135
No. of rats	6	7	30
No. with cecitis	0	0	25
% with cecitis	0	0	83

than in 1940 since the frequency in 4-5 months rats was now 83% as against 44% in the previous study. Furthermore, many of the animals showed very advanced lesions consisting of huge masses made up of glands, cysts containing clear fluid, omentum and thickened cecum. On opening the cecum the characteristic huge ragged ulcers were found. Cultures made from a number of the lesions all yielded bacteria of the *Salmonella* group in large numbers. These organisms showed the following reactions:

glucose +	inosite —	dulcitol +
lactose —	arabinose +	mannitol +
saccharose —	xylose +	urea —
		indol —

and they were considered to fall in the enteritidis group. Cultures from the cecums of several unaffected rats yielded no bacteria of the *Salmonella* group.

The sensitivity of the *Salmonella* bacilli isolated from cecitis was tested with streptomycin, and the organisms were found to be completely inhibited by 5 µg per cc of culture medium. In view of this finding the following observations were made to see if streptomycin had any therapeutic effect.

Experiment I. Twenty male rats were placed on the stock diet of the laboratory. On April 8, 1948, when the rats were about

¹ Bloomfield, A. L., and Lew, W., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 363.

² Bloomfield, A. L., and Lew, W., *ibid.*, 1942, **51**, 28.

³ Bloomfield, A. L., and Lew, W., *ibid.*, 1942, **51**, 179.

⁴ Bloomfield, A. L., *ibid.*, 1943, **53**, 197.

⁵ Bloomfield, A. L., and Lew, W., *Am. J. Med. Sc.*, 1943, **205**, 383.

⁶ Bloomfield, A. L., and Lew, W., *J. Nutrition*, 1943, **25**, 427.

would remain on the table, thrombosis of veins, dislodgement of the plastic tube, and inconstancy of rate of flow. Water and dog biscuits were placed in the cages of the animals receiving fluids intermittently but not in the cages of those receiving constant parenteral alimentation.

Results. Five of the 10 animals died between 2 and 6 days, 2 of electrolyte imbalance, 1 of intussusception with gangrene and necrosis of the distal turned-in end, 1 of leakage of the suture line with a resulting peritonitis, 1 of distemper. The remaining 5 lived between 16 and 45 days with an average survival time of 27.6 days. One died suddenly at 16 days, and necropsy revealed an intussusception of the distal turned-in end with gangrene and necrosis. Another was re-operated at 21 days, while in good condition, and the intestinal continuity restored. The remaining 3 dogs survived for 24, 32, and 45 days respectively, and death was due to emaciation and resulting complications.

That the length of survival was closely correlated with the adequacy of parenteral alimentation was indicated by the animal which lived for 45 days. This animal was maintained in electrolyte balance throughout the course of survival. The average daily intake was 1,091 cc and output 722 cc. Hypoproteinemia and anemia became marked around the fifth and sixth weeks. The animal lost 26% of the initial body weight and was extremely emaciated at death. For the first 10 days, due to much technical difficulty with the plastic tubing, an average of 140 cc of milk daily was allowed and taken orally. Thereafter nothing was allowed by mouth except 10.0 cc of a vitamin mixture daily.

It will be noted (Table I) that only during the last 15 days were we able to administer parenterally sufficient nitrogen and calories to even approach a level adequate to maintain nitrogen balance,⁶ and even at this level the animal continued to lose an average of 0.7% of the body weight daily. However,

⁶ Riegel, C., Koop, C. E., Grigger, R. P., Rhoads, J. E., and Bullitt, L., *S. Clin. N. Am.*, 1945, 1096-1105.

TABLE I.

Dog No. 10	Length of survival 45 days	Time	Avg daily					
			Intake, cc	CrO, g/kg	N, g/kg	enl./kg	NaCl, mg	Output, cc
	First 10 days		881	4.36	0.14	22.45*	4.81	396
	Second 10 days		958	5.15	0.18	23.86	5.58	745
	Third 10 days		1,230	7.20	0.23	32.68	7.55	834
	Last 15 days		1,226	7.76	0.33	37.20	6.78	850
							kg	Weight loss, %
							0.06	0.4
							0.12	0.8
							0.07	0.5
							0.09	0.7
								Temp.
								1024
								1030
								1026
								1030

* Includes calories from fat in milk administered for first 10 days.

of scarring and adhesions, not a single open ulcer was found in the treated rats. The untreated animal (No. 76498) on the other hand showed the typical changes of active cecitis with a large ulcer.

Summary. The lesions of ulcerative cecitis of rats resolved within 10 days of streptomycin treatment. The antibiotic was added

to the animals' drinking water in an average dose of 0.1 g per day per rat. The bacilli of the *Salmonella* group which are invariably associated with the ulcers of cecitis were sensitive to streptomycin in the test tube and they could no longer be grown from the cecums of treated rats with healed lesions.

16600 P

Simple Intestinal Obstruction: Prolongation of Life for 45 Days with Parenteral Alimentation.

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(Introduced by I. S. Ravdin.)

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The average length of survival of animals having a low simple obstruction of the ileum when allowed fluids by mouth or when given saline parenterally is around 8 to 14 days.^{1,2} Death has been attributed to loss of fluid and electrolytes, possible nervous effects of distension, or the absorption of toxins from the obstructed bowel. By administering fluids, electrolytes, and nutritive material into an enterostomy below a high simple intestinal obstruction, Jenkins^{3,4} has maintained animals for from 50 to 70 days. No cause for death other than the long standing emaciation was found.

We have studied the course and length of survival of animals having a simple obstruction of the ileum in which electrolyte imbalance, dehydration, anemia, and starvation were combatted by the intensive administration intravenously of sodium chloride, 5 or

10% glucose, casein hydrolysate,* blood, plasma, gelatin, and vitamins in an effort to see if by parenteral administration obstructed animals could be maintained as long as those fed by enterostomy.

Method. Ten healthy adult dogs weighing between 10.0 and 15.0 kg were used. The mid-ileum was obstructed by severing the bowel and closing the ends. Postoperatively fluids were administered daily either by intermittent rapid drip intravenously with the animal on the table or, in the later experiments, by constant intravenous drip through a capillary plastic tube threaded into the jugular vein or inferior vena cava after the method of Rhode, Parkins and Vars,⁵ except that a deBaakey pump was not used. The amounts of fluids and calories to be administered were determined by the output (urine and vomitus) and chemical studies on the blood, but it was not always possible to meet the requirements because of the limitations of the methods used for administration; namely, the length of time the animal

¹ Harper, W. H., and Lemmer, R. A., *Bull. Johns Hopkins Hosp.*, 1946, **79**, 207.

² Elman, R., and Hartman, A. F., *Surg., Gynec. and Obst.*, 1931, **53**, 307.

³ Jenkins, H. P., *Proc. Soc. Exp. Biol. and Med.*, 1930, **28**, 111.

⁴ Jenkins, H. P., and Beswick, W. F., *Arch. Surg.*, 1933, **26**, 407.

* Amigen 5% in 5% dextrose solution made by Mead Johnson & Company.

⁵ Rhode, C. M., Parkins, W. M., and Vars, H. M., *Bull. Am. Coll. Surg.*, 1947, **32**, 255.

TABLE I.
Effect of Gastric Mucin, Egg Yolk, and Saline Diluents on Virulence of Tubercle Bacilli (H37Rv) for C3H and CFW (Albino) Mice.

Mouse strain	Diluent	No. of mice	No. with macroscopic lesions	% liver lesions	% peritoneal and lymph node lesions
C3H	Mucin	18	18	39	88
"	Egg yolk	18	10	40	60
"	Saline	16	0	0	0
CFW	Mucin	15	2	0	100
"	Saline	12	0	0	0

and saline for C3H and CFW (albino) mice are summarized in Table I. The mice were inoculated intraperitoneally with 0.5 cc of each mixture composed of equal parts of tubercle bacilli suspension and diluent. Each mouse was inoculated with approximately 500,000 bacilli. All animals were sacrificed 10 days after inoculation and autopsied for evidence of macroscopic lesions. Impression smears were routinely made of gross lesions and stained by the Ziehl-Neelsen technic. Lesions varied in size from 2 to 3 mm and usually contained large numbers of tubercle bacilli in impression smears. When present lesions were seen at the site of inoculation, liver, spleen or intestinal lymph nodes. A few tubercle bacilli were usually found in impression smears of the spleen or liver of mice inoculated with mucin suspensions, even if macroscopic lesions were not present in these organs. No gross lesions were seen in the lungs. Similar results were obtained using dba mice and recently isolated tubercle bacilli.

Since the above experiments were completed we have made several (14) isolations of tubercle bacilli from patients 10 to 15 days after inoculation by the use of pigmented mice (C3H or dba) and mucin. A few of the positive results in mice were obtained with microscopically negative sputum. The patient's sputum or feces were prepared in the usual manner, mixed with equal parts

of 5% mucin, and inoculated both subcutaneously and intraperitoneally (0.5 cc) into each of 4 or 5 mice. Tubercle bacilli were readily recovered from a number of the lesions planted in Petraghini's medium. Impression smears of the spleen, liver and peritoneal fluid were routinely made of all inoculated mice because acid-fast bacilli could sometimes be demonstrated although no macroscopic lesions were present. Comparative studies are now in progress in which routine specimens from patients are being inoculated into mice, guinea pigs and culture media simultaneously.

Summary. Gastric mucin significantly enhances the virulence of tubercle bacilli for pigmented strains (dba, C3H or C57 black) of mice. Grossly visible lesions containing large numbers of tubercle bacilli are usually present 10 days after intraperitoneal inoculation with the dosages and strains of tubercle bacilli studied. Several isolations of tubercle bacilli from patients have been made 10 to 15 days after inoculation by use of pigmented mice (dba or C3H) and mucin.

Addendum. Recently the number of isolations from patients has been increased by feeding pigmented mice inoculated with mucin the cornmeal-gelatin-butter diet of Dubos and Pierce.⁴

⁴ Dubos, R. J., and Pierce, C., *Am. Rev. Tuberculosis*, 1948, 57, 287.

despite the inability to maintain adequate nutrition, and in the face of intermittent, severe vomiting (1500 to 2500 cc every 3-4 days) and a sustained elevated temperature due to thrombosis of veins resulting from the methods used, this animal was maintained for 45 days, and during the last 35 days all fluids were administered intravenously.

Conclusions. The length of survival of

dogs having a low simple intestinal obstruction depends upon the ability to maintain fluid, electrolyte, and nutritional requirements. By parenteral alimentation with the methods used adequate nutritional requirements were not obtained, but in 1 animal in which these requirements were approached more closely the length of survival was 45 days.

16601 P

A Rapid Mouse Test for Laboratory Diagnosis of Tuberculosis.*

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Recent reports have shown that pigmented strains of mice (dba, C3H and C57 black) are more susceptible to tuberculosis than albino mice.^{1,2} We have confirmed these findings and have completed additional experiments in which albino and pigmented mice were inoculated intraperitoneally with tubercle bacilli suspended in various diluents. This was done with the hope of finding an enhancing agent for a rapid diagnostic test for tuberculosis.

This paper is a preliminary report on the use of gastric mucin for enhancing the virulence of the H37Rv and several recently isolated strains of mammalian tubercle bacilli for pigmented mice. Although there have been no previous reports to our knowledge on the inoculation of mice with mucin suspensions of tubercle bacilli, Mills and Colwell³ showed that guinea pigs inoculated sub-

cutaneously with small doses of tubercle bacilli suspended in mucin developed larger local lesions than controls inoculated with saline suspensions. These workers found no difference in pathogenicity when larger dosages were inoculated. More recently Pierce *et al.*² have shown that the infectivity of tubercle bacilli for mice is enhanced by egg yolk.

For the present studies CFW albino mice and C3H, dba and C57 black strains of pigmented mice weighing about 13 g were used. The virulent human type tubercle bacillus H37Rv or recently isolated strains from cases of tuberculosis were studied. Suspensions were prepared from 3-4-week-old cultures grown on Petragnini medium. The organisms were suspended in physiological saline and clumps were dispersed by means of glass beads. The suspension was adjusted to approximately 1 million organisms per cc by a nephelometer tube, and the count was checked with a Petroff-Hausser counting chamber. Mucin was prepared by suspending 5 grams of granular mucin (Type 1701-W, Wilson lab.) in 100 cc of distilled water, adjusted to pH 7.4 and autoclaved. Egg yolk was obtained aseptically and diluted with equal parts of physiological saline.

Results comparing the virulence of the H37Rv strain suspended in mucin, egg yolk

* Aided by the Sheila Kelman Memorial Fund. Supported in part by the Michael Reese Research Foundation. We are indebted to Sylvia King and Phyllis Conner for technical assistance.

¹ Long, E. R., and Vogt, A. B., *Am. Rev. Tuberculosis*, 1941, **44**, 196.

² Pierce, C., Dubos, R. J., and Middlebrook, G., *J. Exp. Med.*, 1947, **86**, 159.

³ Mills, M. A., and Colwell, C. A., *Am. Rev. Tuberculosis*, 1939, **40**, 109.

lymph nodes have been reported elsewhere¹⁵ and will not be repeated here. On examination of the sections of normal nodes by methyl-green and pyronine, almost no pyronine stained material could be found.

The histologic differences among the stimulated nodes were primarily those of degree. In these nodes, one day after the injection of antigen, a few young lymphocytes were seen to contain red granules in the cytoplasm, and in many of these cases, clearly defined red nucleoli. Such cells were progressively more numerous on the second, third and fourth days. On these latter days there was an occasional crescentic accumulation of pyronine-staining material at the very edge of the nucleus of cells which showed this material, and occasionally cells were seen which showed no cytoplasm except for a sector of material solidly stained red, contiguous to about one-quarter of the circumference of an otherwise denuded nucleus.

No plasma cells were seen in the greatly enlarged cortex of the stimulated lymph nodes by either method of staining. The few plasma cells noted were in the medullas of lymph nodes, which were fairly similar in area to those of control nodes, and the number of these cells did not differ remarkably, in these preparations, between control and stimulated nodes.

The great majority of cells with pyronine-staining granules were young lymphocytes. A few more mature, smaller, lymphocytes showed these granules, on the third and fourth days, and a number of the cells with these

granules had nuclei so vesicular as to suggest that they were transitional forms between the reticulum cells on one hand and clearly recognizable young lymphocytes on the other. Within this range the distribution was continuous: A complete series of cells with pyronine-staining cytoplasmic granules could be picked out of a given section, from transitional forms to mature lymphocytes. Large lymphocytes of various stages were certainly much more in evidence than the mature cells among those with ribonucleic acid granules. ¹¹

It is well to recall that the pyronine-staining granules are not the new protein, or antibody, itself, but a part of the mechanism which produces this protein. Accordingly it is quite consistent with our interpretation that the ribonucleic acid granules are found primarily in young rather than old lymphocytes.

Summary. The identification of the cell within lymphatic tissue which produces antibody has been approached by the application of a new histochemical technic for localization of cytoplasmic ribonucleic acid. The latter has been found to be an invariable concomitant to the formation of new protein in tissue. In lymph nodes actively engaged in the production of antibodies a wide range of lymphocytes, largely younger forms, was found to have cytoplasmic granules and nucleoli stained with pyronine, which is used to identify ribonucleic acid. Such granules and nucleoli were found also in transitional forms between reticulum cells and lymphocytes, but in no other cell types.

16603

Viscosity Studies of Erythrocytes from Persons with Sick Cell Disease.

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The viscosity of the erythrocytes of sickle cell disease varies with changes in oxygen tension as Ham and Castle^{1,2} have shown. The variation in viscosity is due to changes in

¹ Ham, T. H., and Castle, W., *Trans. Assn. A. Phys.*, 1940, 55, 127.

² Ham, R. H., and Castle, W., *J. Clin. Invest.*, 1940, 19, 788.

Histologic Evidence for the Synthesis of Protein in Lymphocytes Following Parenteral Injection of Antigen.

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Experimental evidence for the formation of antibodies in the local lymphatic system following the parenteral injection of antigen was presented by McMaster, *et al.*,^{1,2} and by Ehrlich and Harris.³ Dougherty, Chase and White⁴ demonstrated antibodies in lymphocytes, and Harris, *et al.*,⁵ showed that lymphocytes were a primary site of antibodies following the local injection of antigens. Other cells of mesodermal origin have, however, been regarded as the possible sources of antibodies: macrophages⁶ and, more recently, plasma cells.^{7,8}

The recent applications of histochemistry to physiology have offered an additional approach to this problem. It was first shown by Caspersson^{9,10} and Brachet¹¹ that cells which were actively forming new protein were characterized by large amounts of ribonucleic acid in their cytoplasm. Additional evidence for the association of cytoplasmic ribonucleoprotein with protein synthesis has been reviewed by Greenstein¹² and by Dempsey and

Wislocki.¹³ Since there is considerable immunologic evidence of the production of antibody by the popliteal lymph node of the rabbit^{3,14,15} following the injection of antigens in the foot pad, and since a tissue in which antibody is being formed could be expected to show evidences of protein synthesis, it seemed feasible to attempt to identify the cells involved by histochemical methods.

Accordingly, a study was undertaken of the localization and concentration of ribonucleic acid in the cells of the popliteal lymph node, in correlation with the production of antibody by that node, after the injection of antigens into the local tissue. This preliminary report will present a brief description of the histologic changes in the lymph node during the first few days after the injection of the antigen.

Methods. Injections of influenza virus into the pads of rabbit feet and the collection of regional (popliteal) lymph nodes were made as described elsewhere.¹⁵ Each animal in this series received the antigen only in one foot, so that the contralateral lymph node could serve as a control specimen. Animals were sacrificed at 1, 2, 3 and 4 days after the injection of antigen. Both popliteal lymph nodes were excised. Parallel sections of each node were stained by Azur II-Eosin and methyl-green-pyronine respectively. The latter set of stains is for the respective nucleic acids: methyl green stains desoxyribonucleoprotein a somewhat bluish green, and pyronine stains ribonucleoprotein red.

Results. The histological findings in the Azur-Eosin stained sections of these popliteal

¹ McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1935, **61**, 783.

² McMaster, P. D., and Kidd, J. G., *J. Exp. Med.*, 1936, **66**, 73.

³ Ehrlich, W. E., and Harris, T. N., *J. Exp. Med.*, 1942, **76**, 335.

⁴ Dougherty, T. F., Chase, J. H., and White, A., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 295.

⁵ Harris, T. N., Grimm, E., Mertens, E., and Ehrlich, W. E., *J. Exp. Med.*, 1945, **81**, 73.

⁶ Sabin, F. R., *J. Exp. Med.*, 1939, **70**, 67.

⁷ Bjoerneboe, M., Gormsen, H., and Lundquist, F., *J. Immunol.*, 1947, **55**, 121.

⁸ Fagraeus, A., *J. Immunol.*, 1948, **58**, 1.

⁹ Caspersson, T., *Naturwissenschaften*, 1941, **20**, 33.

¹⁰ Caspersson, T., *Chromosoma*, 1941, **1**, 605.

¹¹ Brachet, J., *Arch. Biol. Paris*, 1942, **53**, 207.

¹² Greenstein, J. P., *Advances in Protein Chemistry*, 1944, **1**, 210.

¹³ Dempsey, E. W., and Wislocki, G. B., *Physiol. Rev.*, 1946, **26**, 1.

¹⁴ Ehrlich, W. E., and Harris, T. N., *J. Exp. Med.*, 1946, **83**, 373.

¹⁵ Harris, S., and Harris, T. N., in press.

TABLE III.
Effect of Change of pH. Phosphoric Acid Added.

Subject	After O ₂		After CO ₂		Cell count in millions
	pH	Time in secs.	pH	Time in secs.	
18 (S. C. A.)	7.8	160	6.35	221	2.5
Acid added	6.25	138	6.10	192	2.0

TABLE IV.
Effect of Carbon Monoxide.

Subject	Time in secs.			Cell count in millions
	After O ₂	After CO ₂	After CO	
10 (a)	132	147	133	2.0
(b)	106	112	107	1.4

TABLE V.
Effect of Repeated Washing of Cells with Tyrode's Solution and Normal Saline.

Subject	Solution	Time in secs.		Cell count in millions
		After O ₂	After CO ₂	
19 (S. C. A.)	Tyrode's, 3 X	160	195	1.9
	Tyrode's, 20 X	112	130	1.7
	Normal saline, 20 X	119	134	2.4
	On ice—24 hr			
	Normal saline, 20 X	111	120	1.2

TABLE VI.

Subject	Time in secs.		Cell count in millions
	After O ₂	After CO ₂	
20 (a)			
S. C. A. (Heparin)	128	145	2.3
(b) (Oxalate)	123	140	2.0
(c) (NaCN added to b)	126	140	2.0

apparent viscosity of blood will vary with the procedure used for its determination. The size of the capillary and variations in pressure are important in the capillary tube method. The speed of rotation is an important variable in the concentric ring method. In this particular investigation an Ostwald viscosimeter with a relatively wide bore was used so that time intervals would be short and there would be a minimum of sedimentation of red cells. Actual viscosities were not calculated and differences are indicated by varying time intervals.

The viscosity of blood depends upon a number of factors related to the composition of blood itself, most important of which are the protein content of the plasma and the number and character of the erythrocytes. Red

cells separated from plasma and resuspended in Tyrode's solution display viscosity changes due only to changes in the characteristics of the red cells.

Changes in viscosity of suspensions of red cells from persons with sickle cell disease are easily demonstrable and are compared in Table I with normal cell suspensions. It will be seen that such viscosity changes occur in cell suspensions from subjects with sickle-mia (S.C.T.) as well as from subjects with sickle cell anemia (S.C.A.)

There are compared, in Fig. 1, the viscosity measurements of cell suspensions from normal individuals and subjects with sickle cell disease, after exposure to oxygen and to carbon dioxide. It is shown that the viscosity of sickle cells differs from that of normal cells

the red cell itself and not to influences of the suspending medium.³

It was the purpose of this study to investi-

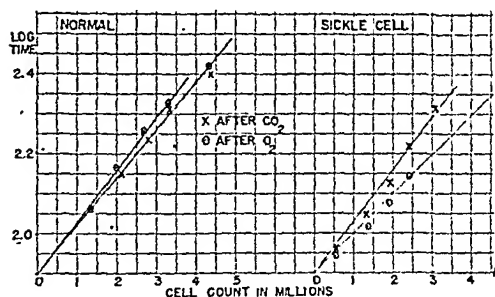


FIG. 1.

Log. of time of flow in seconds plotted against red cell count in millions, after exposure to O_2 and CO_2 .

gate the possibilities of the viscosimetric method as an objective means of measuring the sickling tendency of the red cells of individuals or groups of individuals under varying conditions.

Methods. An Ostwald viscosimeter with

a capillary bore of 0.48 mm in diameter and a length of 7.18 cm was used. The upper reservoir of the viscosimeter was calibrated at a volume of 2 ml. The viscosimeter was suspended in a water bath at 37° C.

Freshly drawn oxalated blood was centrifuged and the erythrocytes washed 3 times in Tyrode's solution. Three ml of cell suspension in Tyrode's solution was placed in the lower reservoir. The appropriate gas was passed through the suspension to effect cell saturation. The time of flow through the capillary was measured with a stop watch.

Results. In Fig. 1 the viscosities of various cell suspensions from a normal subject (No. 1) and from a subject with the sickle cell trait (No. 7) are shown. Viscosity, expressed as the logarithm of the time in seconds, is plotted against the cell count in millions.

Tables I-VI show the results of various studies of blood subjects with sickle cell disease.

TABLE I.
Viscosimeter Time Intervals and Cell Counts for Red Cell Suspensions.

Subject	After O_2	After CO_2	Time difference in seconds	Cell count in millions
1 (Normal)	217	207	- 10	3.9
2 "	233	229	- 4	3.5
3 "	152	151	- 1	2.3
4 "	262	253	- 9	4.5
5 "	271	259	- 12	4.3
6 "	244	228	- 16	4.6
7 (S. C. T.)	192	272	+ 80	4.4
8 (S. C. T.)	236	498	+262	3.8
9 (S. C. T.)	197	321	+124	3.1
10 (S. C. T.)	132	151	+ 19	2.0
11 (S. C. T.)	150	185	+ 35	2.4
12 (S. C. A.)	182	267	+ 85	2.2
(A) (Year later)	139	200	+ 61	2.0
13 (S. C. A.)	141	157	+ 16	2.1
(A) (Year later)	139	200	+ 61	2.0
14 (S. C. A.)	189	281	+ 92	4.2
15 (S. C. A.)	152	175	+ 23	2.1
16 (S. C. A.)	202	320	+116	2.5

TABLE II.
Effect of Nitrogen

Subject	Time in seconds		
	After O_2	After N_2	After CO_2
17 (S. C. A.)	152	182	186

³ Huck, J. G., *Johns Hopkins Hosp. Bull.*, 1923, 34, 335.

Discussion. Several methods for the determination of the viscosity of blood have been used.^{4,5} Both the capillary method and the concentric ring method indicate that the

⁴ Fahreus, R., and Lindquist, T., *Am. J. Physiol.*, 1931, 96, 562.

⁵ Brundage, J. T., *Am. J. Physiol.*, 1934-1935, 110, 659.

obtained from patients suffering from clinical typhus fever⁶ some of which have been preserved by the lyophile process. Any serum showing anticomplementary action was treated by the method of Faran.⁷ It was found that all except one gave positive reaction to both antigens, with titres mostly above 1:320, and one reaching as high as 1:20,480. Among these, 5 sera showed the same titre for both antigens, while 14 sera from 10 patients showed a higher titre to the epidemic antigen: 4 with a 2-fold difference, 4 with 4-fold difference, 5 with 8-fold difference and one positive only to epidemic antigen at titre 1:640.

Comment. The results of the present study by means of complement fixation test agree with a similar study, carried out by one of us (N.C.C.) in the spring of 1946

when epidemic and murine vaccines served as antigens. In that study, 9 patients with 15 samples of sera gave clear cut positive complement fixation reaction with one or both of the antigens. Seven patients with 10 sera gave stronger reaction to the epidemic, one gave equal titre, while another one gave higher titre to the murine antigen. These findings seem to suggest that the strains of rickettsia causing human infections in the past 2 years, whether in the autumn or in the spring, are largely of the epidemic type. This is in contrast to our previous results when the biological study of the organisms tended to classify them as murine type. In order to reconcile the above differences, further study will be made with perhaps absorbed sera. These will be continued when sera from fresh typhus cases will be available.

⁶ We are indebted to Dr. Y. W. Yih, of the Hospital of the National Pei Ta Medical School for 4 of the sera tested.

⁷ Faran, A., *J. Lac. Clin. Med.*, 1946, **31**, 1037.

⁸ Bengtson, I. A., *Pub. Health Rep.*, 1947, **61**, 1379.

16605

Isometric Relaxation Period of the Left Ventricle in Normal Subjects and Patients with Mitral Stenosis.

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Differentiation of a split second sound from reduplication of this sound is important; the latter is consequent to loud snapping of the mitral leaflets in mitral stenosis while the former has a variety of causes, the most common of which is asynchronous closure of the aortic and pulmonic valves. Important information bearing on this problem may be afforded by study of the duration of the period of isometric relaxation of the left ventricle, for this period is the interval between closure of the aortic valve and opening of the mitral. Several attempts have been made to measure this period in normal subjects by means of arterial and venous tracings¹ or by observation of roentgenological tracings (electroky-

mograms).² The phonocardiogram has been used, thus far, for measuring this interval only in patients with mitral stenosis.³ Errors due to delay in the transmission of the *v* wave from the right heart to the neck or asynchronism between right and left ventricles cannot be avoided in studies based on mechanical methods; similarly, inaccuracies due to the influence of position and motion phenomena may be present in roentgenological tracings. However, the phonocardiogram gives a virtually instantaneous recording of the vibration due to valvular movements and is

² Randak, E. F., Boone, B. R., Ellinger, G. E., and Oppenheimer, M. J., *Fed. Proc.*, 1948, **7**, 97.

³ Margolies, A., and Wolferth, C., *Am. Heart J.*, 1932, **7**, 443.

¹ Burststein, J., *Am. J. Phys.*, 1923, **65**, 158.

after treatment with carbon dioxide. Increase in viscosity with nitrogen is shown in Table II.

Variations of the hydrogen ion concentration do not seem to affect the viscosity of the abnormal cells (Table III).

Carbon monoxide reacts with hemoglobin in much the same way as oxygen although a far more stable compound, carbon monoxide hemoglobin, is formed. Murphy and Shapiro⁶ found that exposure of cells from a subject with sickle cell anemia to carbon monoxide apparently prevented their sickling. In order to confirm this, cells which had the sickling tendency were treated with carbon monoxide and studied in the viscosimeter. The sickling process was inhibited by this treatment as it was no longer possible to produce viscosity changes with carbon dioxide

or nitrogen (Table IV).

Tomlinson and Jacob⁷ report an inhibition of sickling by repeated washings and by the addition of cyanides to sickle cell blood. It could not be demonstrated by the viscosimetric method that repeated washings inhibited the sickling tendency (Table V). The addition of cyanide or the use of various anticoagulants showed no influence on the sickling tendency as viscosity changes could still be demonstrated after their use (Table VI).

Summary. A viscosimetric method is described for the study of the sickling tendency of red cells from subjects with sickle cell disease. This method does not differentiate between sickle cell anemia and sickle cell trait. Data are compared with observations of other investigators.

⁷ Tomlinson, W. J., and Jacob, J. E., *J. Lab. and Clin. Med.*, 1945, 30, 107.

⁶ Murphy, R. C., and Shapiro, S., *Ann. Int. Med.*, 1945, 23, 376.

16604 P

Studies of Typhus Fever in Peiping by the Complement Fixation Test.

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Recently, the complement fixation reaction in typhus fever, first studied by Castaneda,¹ has been refined so that not only typhus may be differentiated from other rickettsial diseases such as Rocky Mountain Spotted Fever,² but may itself be classified according to epidemic or murine nature.³ Inasmuch as we have been interested in the etiology of typhus fever in this area, and have reached certain tentative conclusions based on the studies of the biology of the rickettsia strains locally isolated,⁴ we wish now to use the

complement fixation test to complete our studies. Unfortunately so far, owing to the scarcity of clinical cases seen in the past year, the number of sera examined has been small. But the results seem to be sufficiently consistent to warrant a preliminary report at this time.

Method and results. The antigens used in the present study, kindly supplied by the Lederle Laboratory Division of the American Cyanamid Company,⁵ consist of washed rickettsial bodies. The method of complement fixation test is that of Kolmer's modified by reducing all ingredients to one-half the volume of the regular method. Sera were

¹ Castaneda, M. R., *J. Immunol.*, 1936, 31, 285.

² Bengtson, I. A., *Am. J. Pub. Health*, 1945, 35, 701.

³ Wishart, F. O., and Malcomson, M. E., *Canad. Pub. Health J.*, 1946, 37, 369.

⁴ Liu, W. T. et al., *Am. J. Hyg.*, 1942, 35, 231.

⁵ We are indebted to Dr. E. B. Watson, of the International Health Division of the Rockefeller Foundation for securing these antigens.

TABLE I.

	Distance between main phase of 2nd sound and opening sound (or snap) of the mitral valve		Distance between the two phases of the split 2nd sound	Distance between 2nd and 3rd sounds
	Normal subjects	Mitral stenosis		
Maximum	0.07	0.11	0.05	0.18
Minimum	0.04	0.07	0.025	0.12
Average	0.055	0.079	0.04	0.15

viously.^{5,6,7} In our collection of fluorocardiograms, 32 tracings were recorded in patients with mitral stenosis; one of the latter had a pronounced snap of the mitral valve (Fig. 2).

Results. The results are presented in Table I, in seconds.

The normal subject presenting a visible opening sound in the phonocardiogram, on whom a fluorocardiogram was recorded, showed the following:

(a) The interval between the main vibration of the second sound and the opening sound was 0.07 seconds.

(b) The tracing of the ventricle exhibited an early diastolic rebound.

(c) While the negative peak of the ventricular tracing coincided with the main vibration of the second sound, the opening sound coincided with the end of the initial rise and preceded the rebound (Fig. 1). Similar findings were present in tracings recorded along the left ventricular margin.

The patient with mitral stenosis and an opening snap of the mitral valve on whom a fluorocardiogram was recorded showed the following:

(a) The interval between main vibration of the second sound and opening snap was 0.11 second.

(b) The tracing of the left ventricle presented an early diastolic rebound.

(c) The relationship between opening snap and rebound is identical with that of the normal subject in spite of the prolongation of the isometric relaxation period.

Discussion. Our average figures for the duration of isometric relaxation in normal subjects are somewhat less than those found by Burstein.¹ This can be explained by the greater accuracy of our method which excluded transmission time of mechanical waves from the heart to the neck and results of phenomena taking place in both ventricles; moreover, our data were obtained by the use of a speed film. On the other hand, our findings coincided with those reported by Hoff.⁸ Our data concerning measurements in patients with mitral stenosis are similar to those of Margolies and Wolferth³ in regard to the average figures. Their minimum and maximum figures are respectively smaller and larger than ours, but they were found during extreme alterations of the cardiac rate. Our data on the interval between the two phases of a split second sound are shorter than those of Wolferth,⁹ possibly because of a different choice of cases, while those concerning the third sound are identical with his. There is a slight difference between our data concerning the third sound and those of Rappaport and Sprague.⁴ This difference can be explained by the fact that these authors measured the interval from the beginning of the second sound.

Simultaneous observation of the phono-

⁵ Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., *Am. Heart J.*, 1948, **35**, 336 and 348.

⁶ Luisada, A. A., and Fleischner, F. G., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 436.

⁷ Fleischner, F. G., Romano, F. J., and Luisada, A. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 535.

⁸ Hoff, H. E. Events of Cardiac Cycle. In *Howell's Textbook of Physiology* by Fulton, Saunders, Philadelphia, 1946.

⁹ Wolferth, C. C. Heart Sounds, in *Cyclopedia of Medicine*, Vol. 3. F. A. Davis, Philadelphia.

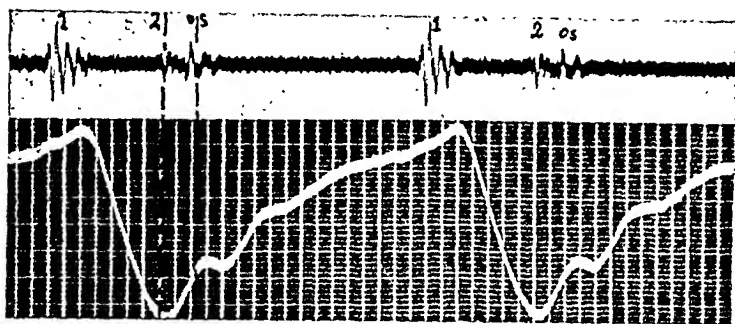


FIG. 1.

Simultaneous phonocardiogram and fluorocardiogram in a normal subject. The former has two vibrations indicating the closing of the semilunar valves (2) and the opening of the mitral valve (os). Between them lies the isometric relaxation period.

independent of other causes of error; it is therefore, the most accurate method. Thus far, only data obtained in cases of mitral stenosis are available³ because, even if recognition of the various components of the second sound is often possible,⁴ the vibration due to opening of the mitral valve can be recognized only in a small number of normal subjects. However, the systematic study of a series of over 1500 phonocardiograms collected by one of us (Luisada) showed the possibility of clearly isolating the vibration due to the opening of the mitral valve in a small percentage of cases. This vibration will be called "opening sound of the mitral valve".

Materials and Methods. This study is based on the above mentioned collection of phonocardiograms as well as the collection of fluorocardiograms and phonocardiograms of the Beth Israel Hospital. The records of seven normal subjects permitted measurement of the isometric relaxation period of the heart on the basis of the phonocardiogram. One of these cases also had a fluorocardiogram (electrokymogram) recorded and, therefore, permitted the analysis of the latter tracing on the basis of the phonocardiographic study (Fig. 1). Twenty-four cases had mitral stenosis with an evident opening snap of the mitral valve. This permitted the determination of the isometric

relaxation period of the left ventricle in rheumatic heart disease where there is a possibility of delayed opening of the left auricular-ventricular valve. Parallel studies were made in 9 cases of mitral stenosis with a split second sound at the base, in 19 subjects having a similar splitting without mitral stenosis, and in 20 cases with a third heart sound. The phonocardiograms were recorded by means of a Sanborn "Stethocardiette", using a stethoscopic microphone with a 3.7 cm funnel ap-

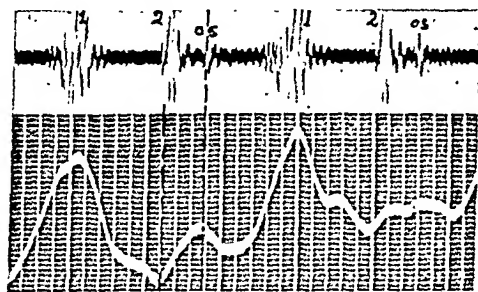


FIG. 2.

Simultaneous phonocardiogram and fluorocardiogram of a patient with mitral stenosis and auricular fibrillation. The sound tracing presents a loud opening snap of the mitral valve (os). Between the main vibration of the second sound (2) and the opening snap (os) is the isometric relaxation period which is prolonged.

plied at the apex, at the third left interspace, and at the second left interspace. The fluorocardiogram is a tracing of the movements of the cardiovascular fluoroscopic silhouette recorded by a phototube and a galvanometer. Details of this method have been given pre-

⁴ Rappaport, M. B., and Sprague, H. B., *Am. Heart J.*, 1942, 23, 591.

the toxic dose and the therapeutic dose in mice which had been infected with *Diplococcus pneumoniae*, type 1. Sulfacin is active against gram-positive microorganisms, primarily, and in this respect it resembles penicillin. We were interested to learn if a culture of a gram-positive microorganism such as *Staphylococcus aureus* readily acquired an increase in its resistance to sulfacin, whether a sulfacin-resistant strain of staphylococci showed any change in its susceptibility to penicillin, and whether a penicillin-resistant strain of staphylococci showed any increase in its resistance to sulfacin.

The supply of pure sulfacin was that mentioned previously.² The commercial sodium salt of penicillin was used. Stock solutions of both antibiotics were prepared in sterile distilled water and stored in the dry-ice box. Bacto-nutrient broth, adjusted to pH 7.4 was employed as the culture medium.

Penicillin-resistant and sulfacin-resistant staphylococcal cultures were developed *in vitro* by growing the test organism in the presence of varying concentrations of the antibiotics. From the stock solutions of each antibiotic serial two-fold dilutions were prepared in Bacto-nutrient broth, pH 7.4. All of the dilution tubes were then seeded with one drop of broth culture of *Staphylococcus aureus* and the tubes incubated at 37°C. Observations were usually made after 24 hours, but in some instances the incubation period was 48 or 72 hours. The tubes in the penicillin series and in the sulfacin series containing the highest concentration of the antibiotic which showed visible growth of

staphylococci were selected for subculturing. New series of dilutions of penicillin and sulfacin were prepared and inoculated from the tubes in the previous series which had been selected for subculturing. This procedure was repeated until penicillin-resistant and sulfacin-resistant cultures of staphylococci were obtained. The extent of the tolerance of the cultures for the two antibiotics is shown in Table I. Parallel transfers of the parent culture of staphylococci were made in the Bacto-nutrient broth for a control.

Tests to determine the sensitivity to penicillin and to sulfacin were then made with the penicillin-resistant strain, the sulfacin-resistant strain and the normal culture of staphylococci. In becoming resistant to penicillin the staphylococcal culture did not change in its sensitivity towards sulfacin. Likewise when the culture of staphylococci became resistant to sulfacin it did not change in its sensitivity towards penicillin. Typical results of repeated tests are presented in Table II.

Results. After 7 serial transfers of a staphylococcal culture in Bacto-nutrient broth containing varying amounts of sulfacin, the culture grew in the presence of 10 µg of the drug. This represents an increase in resistance of the culture to the drug of over 1100 times that of the original strain (Table I). Under similar conditions the staphylococcal culture developed a resistance to penicillin of slightly over 32 fold. The acquisition of a tolerance for either penicillin or sulfacin by the staphylococcal culture was not accompanied by an increase in tolerance for the other antibiotic.

Discussion. The value of using drug-fast strains of microorganisms in studying new chemotherapeutic compounds was described by Ehrlich³ and was advocated in the study of new antibiotics by Stansly,⁴ Eisman *et*

* This project has been supported by the Smith, Kline and French Laboratories, Philadelphia, Pa.

† This is based upon a thesis in medical bacteriology presented to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Master of Science.

‡ Present address: School of Tropical Medicine, Government of Puerto Rico, San Juan, Puerto Rico.

¹ Junowicz-Kocholaty, R., Kocholaty, W., and Kelner, A., *J. Biol. Chem.*, 1947, 168, 765.

² Morton, H. E., *PROC. SOC. EXP. BIOL. AND MED.*, 1947, 66, 345.

³ Ehrlich, P., *The Harben Lectures for 1907 of the Royal Institute of Public Health, London*. H. K. Lewis, 1908.

⁴ Stansly, P. G., *Science*, 1946, 103, 402.

cardiogram and fluorocardiogram, both in the case with a physiological opening sound and in that with a pathological opening snap, indicates the following:

The part of the fluorocardiogram which corresponds to the isometric relaxation period

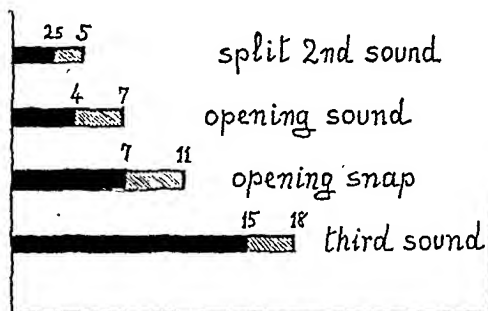


FIG. 3.

Scheme indicating the distance in hundreds of seconds between the two phases of a split or reduplicated second sound and between second sound and third sound. The black bar indicates the minimal distance, the striped bar, the maximal distance between the two phases of the reduplicated sound or between second and third sound. *Split second sound*, recorded over the pulmonic area. *Opening sound*, recorded over the mitral area in normal subjects. *Opening snap*, recorded over the apex or mitral area in patients with mitral stenosis. *Third sound*, recorded over the apex in normal subjects.

lies between the lowest point of the ventricular wave and the beginning of the rebound, and not that ending with the lowest point of the rebound, as suggested by others.¹⁰ How-

¹⁰ Boone, B. R., Chamberlain, W. E., Gilliek, F. G., Henny, G. C., and Oppenheimer, M. J., *Am. Heart J.*, 1947, **34**, 560.

ever, this period cannot be measured routinely on the various fluorocardiographic records because the diastolic rebound is not a constant feature; it may be represented by a less steep rise of the tracing or may be completely absent. According to our data, the figures found recently by others² for the isometric relaxation period are too long, especially because they did not study patients with mitral stenosis; as known, these may present the longest duration of the isometric relaxation period because of impaired opening of the mitral valve.

The results of our study, sketched in Fig. 3, facilitate basing the classification of an extra-sound on the above temporal data.

Summary. The isometric relaxation period of the left ventricle in normal subjects and in patients with mitral stenosis was studied. This period was measured on the phonocardiogram as the interval between the main vibration of the second sound and the opening sound or snap of the mitral valve.

The findings in 7 normal subjects varied between 0.04 and 0.07 seconds; 24 mitral patients had figures of from 0.07 to 0.11 seconds. Additional data on the split second pulmonic sound and the third heart sound are presented.

Simultaneous phonocardiograms and fluorocardiograms in a normal subject and in a patient with mitral stenosis, both having an opening sound or snap of the mitral valve, allowed correlation of the ventricular fluorocardiogram with the sound tracing in regard to the isometric relaxation period.

16606

Staphylococcus aureus: Drug-fastness Studies with Penicillin and Sulfactin.*†

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(Introduced by A. N. Richards.)

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Sulfactin, a new antibiotic produced by an *Actinomyces*, was described by Junowicz-Kocholaty, Kocholaty, and Kelner.¹ Its bacterial

spectrum, toxicity, and therapeutic action in mice were described by Morton.² It was found that there was a very favorable ratio between

et al.,⁵ Graessle and Frost,⁶ and Sullivan *et al.*⁷

Therapeutic failures, as a result of a particular strain of an infecting parasite acquiring or possessing a tolerance to the drug, have been known since the advent of chemotherapy. The superior therapeutic action effected by the use of more than one chemotherapeutic agent was pointed out by Laveran⁸ in his work with trypan red and arsenous acid in experimentally induced trypanosomiasis. The rationale of a plan of drug rotation in the case of certain bacterial infections was pointed out by Feirer, Meader and Leonard⁹ and again by Duemling and Horton.¹⁰

Ungar¹¹ first demonstrated the synergistic effect *in vitro* and *in vivo* of chemotherapeutic agents (para-aminobenzoic acid and sulfapyridine) on an antibiotic (penicillin) and Carpenter, Bahn, Ackerman, and Stokinger¹² pointed out the advantages of combining 3

chemical substances with penicillin in order to prevent the development *in vitro* of drug-fast strains of the gonococcus. Although sulfactin is active against the same types of microorganisms against which penicillin is active, it nevertheless has a possible role in the field of chemotherapy.

It will be noted that the sensitivity of the staphylococcal strain to sulfactin at the beginning of the studies (Table I) is slightly different from that found for the parent culture later in the studies (Table II). A possible factor in explanation of this may be that a different lot of stock solution was employed in the later tests but this is not a complete explanation.

The magnitude of the resistance to sulfactin developed by a strain of staphylococci was intermediate between the increase of the same strain of staphylococci to penicillin and the increase in resistance of a strain of *Escherichia coli* to streptomycin.¹³

Summary. A strain of staphylococci in becoming resistant to penicillin did not show an increase in its resistance to sulfactin. A similar strain of staphylococci in becoming resistant to sulfactin did not show an increase in its resistance to penicillin. The increase in resistance to sulfactin developed by the strain of staphylococci was intermediate between the increment in resistance developed by staphylococci to penicillin and the increase in resistance developed by a strain of *E. coli* to streptomycin.

¹³ Kohn, A., and Morton, H. E., *J. Bact.*, 1947, 53, 695.

⁵ Eisman, P. C., Marsh, W. S., and Mayer, R. L., *Science*, 1946, 103, 673.

⁶ Graessle, O. E., and Frost, B. M., *Proc. Soc. Exp. Biol. and Med.*, 1946, 63, 171.

⁷ Sullivan, M., Stahly, G. L., and Birkeland, J. M., *Science*, 1946, 101, 397.

⁸ Laveran, M. A., *Comp. rend. Acad. Sci.*, 1894, 120, 19.

⁹ Feirer, W. A., Meader, P. D., and Leonard, V., *J. Urol.*, 1926, 16, 97.

¹⁰ Duemling, W. W., and Horton, E. H., *U. S. Naval Med. Bull.*, 1947, 47, 695.

¹¹ Ungar, J., *Nature*, 1943, 152, 245.

¹² Carpenter, C. M., Bahn, J. M., Ackerman, H., and Stokinger, H. T., *Proc. Soc. Exp. Biol. and Med.*, 1945, 60, 162.

16607

Quantitative Chemical Analysis of the Adrenal Glands of Wild Norway Rats.

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Rogers and Richter¹ pointed out that the

¹ Rogers, P. V., and Richter, C. P., *Endocrinology*, 1942, 42, 46.

wild Norway Rat has an adrenal gland 2-3 times as large as that of the domestic albino Norway Rat. This difference in size is limited to the cortex. Since the cortical hor-

TABLE I.
Development of Resistance in Vitro of a Strain of *Staphylococcus aureus* (P 210) to Sulfacin and Penicillin.

Development of resistance in <i>in vitro</i> of a strain of <i>Staphylococcus aureus</i> (F 210) to Sulfacin and Penicillin.																									
Antibiotic	Tube No.	Sulfacin													Penicillin										
		c	1	2	3	4	5	6	7	8	9	10	11	12	13	c	1	2	3	4	5	6	7	8	9
Approximate conc. of anti- biotic per ml of medium	0	10 μ g	5 μ g	2.5 μ g	1.25 μ g	0.625 μ g	0.312 μ g	0.156 μ g	0.078 μ g	0.039 μ g	0.019 μ g	0.009 μ g	0.004 μ g	0.002 μ g	0	10 U	5 U	2.5 U	1.25 U	0.625 U	0.312 U	0.156 U	0.078 U	0.039 U	
	1	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	3	3	3	3
	2	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	3	3	3	3
	3	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	3	3	3	3
	4	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	3	3	3	3
	5	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	3	3	3	3
	6	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	3	3	3	3
7	4	3	3	3	—	+	2	3	4	4	4	3	3	4	4	+	2	3	3	4	—	—	—	—	

— = no growth.

4 = maximum amount of growth.

3 and 2 = proportionate amount

1 = smallest amount of growth readily detected macroscopically.

+

= trace, or growth barely detectable macroscopically.

TABLE II.

Comparison of Typical Results Obtained by Testing the Parent Culture of *Staphylococcus aureus*, P 210, and the Penicillin-adapted and Sulfacin-adapted Strains of This Culture Against Penicillin and Sulfacin.

Antibiotic	Sulfactin									Penicillin										
	c	1	2	3	4	5	6	7	8	9	c	1	2	3	4	5	6	7	8	9
Concentration of antibiotic per ml of medium	0	10 μ g	5 μ g	2.5 μ g	1.25 μ g	0.625 μ g	0.312 μ g	0.156 μ g	0.078 μ g	0.039 μ g	0	10 μ	5 μ	2.5 μ	1.25 μ	0.625 μ	0.312 μ	0.156 μ	0.078 μ	0.039 μ
<i>Staphylococcus aureus</i>																				
Parent culture	4	—	—	—	—	—	—	—	3	4	4	—	—	—	—	—	—	1	2	2
Penicillin-adapted strain	4	—	—	—	—	—	—	—	3	4	4	—	—	—	—	—	—	1	2	3
Sulfactin-adapted strain	4	4	4	4	4	4	4	4	—	—	4	+	+	—	—	—	—	—	—	—

—, 1, 2, 3, and 4 = amount of growth as described in footnote to Table I.

than the cortex of the domestic rat, it is apparent that the wild rat has much more cholesterol available for hormone production than does the domestic rat of comparable size. The writer is unable to offer any explanation for the disparity in the two sets of figures by different investigators shown in the table.

It should be pointed out that these rats were caught in spring steel traps and in some cases may have remained in the trap for as long as 12 hours. The emotional disturbances

and trauma associated with this may have caused a lowering of the original cholesterol.

Summary. Quantitative analysis of the adrenal glands of the wild Norway rat reveals that the total fats comprise 6.5% of the gland and that of this 3.7% are cholesterol. These figures are lower than the values for the adrenal gland of the domestic rat. However, since the cortex of the adrenal gland of the wild rat is 2-3 times larger than that of the domestic rat, the wild rat has much more cholesterol available for hormone production.

16608

Complement Fixation in Human Sera Following Murine Typhus.

E. R. RICKARD.

From the International Health Division, Rockefeller Foundation, and Florida State Board of Health.

Complement-fixing antibodies for murine typhus have been shown to persist in the sera of both human beings and rodents for long periods of time following infection.¹⁻³ The complement fixation test has been employed, therefore, to estimate the past incidence of typhus in human⁴ and rodent⁵ populations. For this reason, an appraisal of the accuracy of the test as an indicator of past infection should be of interest. During a recent state-wide survey of murine typhus in Florida,⁶ clinical and epidemiological records were completed on 2055 persons who, during the years 1944, 1945 and 1946, had had typhus or had been strongly suspected of having had the disease. The present report will

describe the results of complement fixation tests performed upon the sera of 404 persons whose cases were investigated during the survey.

Methods. All persons from whom serum specimens were obtained had had fever and constitutional symptoms sufficiently severe to confine them to bed for a period of at least 10 days. Furthermore, all these persons had had relatively close contact with rats and their ectoparasites, and there was epidemiological evidence that they could have contracted typhus.

Sera were collected aseptically at intervals varying from 7 days to 3 years and 11 months following the onset of illness, and were stored at icebox temperature for periods varying from 7 days to 4 months before examination. Immediately before testing they were inactivated by exposure to a temperature of 56°C for 30 minutes.

Antigen* was prepared by the Cox method⁷ from the yolk sacs of chick embryos infected

¹ Bengtson, I. A., *Pub. Health Rep.*, 1941, 50, 649.

² Bengtson, I. A., and Topping, N. H., *Am. J. Pub. Health*, 1942, 32, 48.

³ Bengtson, I. A., *Am. J. Pub. Health*, 1945, 35, 701.

⁴ Davis, D. E., and Pollard, M., *Pub. Health Rep.*, 1946, 61, 928.

⁵ Davis, D. E., and Pollard, M., *Am. J. Trop. Med.*, 1946, 26, 619.

⁶ Rickard, E. R., and Riley, E. G., *Am. J. Pub. Health*, to be published.

* We are grateful to Dr. Herald R. Cox of the Lederle Laboratories for furnishing the antigens used in this study.

⁷ Cox, H. R., *Pub. Health Rep.*, 1938, 53, 2241.

TABLE I.

	Wt of animal in g	Total fat	Total cholesterol	Source
Wild Norway	250-475	6.5% \pm 1.2*	3.7% \pm 0.8*	This paper
Domestic				
Wistar	300	8.7	5.1	Nichols ²
Sprague-Dawley	200		5.48	Tepperman ³
Long-Evans	(Adult)		5.28	Sperry ⁴
Sprague-Dawley	250		5.84	Sayers <i>et al.</i> ⁵
Yale	24-day-old		3.03	Sayers <i>et al.</i> ⁵
Long-Evans	284		3.45	Levine ⁷

$$* = \pm \sqrt{\frac{\sum d^2}{N}}$$

none is likely formed from the cholesterol present in the gland it would be interesting to compare the cholesterol concentration in the adrenals of the two strains of animals. Such data are not in the literature. Accordingly the following study was carried out.

The adrenal glands of 41 wild Norway Rats* were subjected to a chemical analysis which has been previously described.² These rats were trapped during a typhus survey program currently being carried out in this state. They varied in size from 250 g to 475 g and were about equally distributed between the two sexes. Pregnant females were not used.

Results. The results were tabulated in comparison with the findings of other investigators on the adrenal of the domestic rat.

Discussion. In their paper Rogers and Richter present an interesting discussion based largely on the fact that the lean, aggressive, wild rat has a "faster pace of living" than does the obese, sedentary, domestic rat.

* These rats were kindly made available by Drs. H. F. Schoof and G. C. Barden of the State Health Department, Raleigh, N. C.

² Nichols, J., *J. Aviation Med.*, 1948, 19, 171.

³ Tepperman, J., Tepperman, H. M., Patton, B. W., and Nims, L. F., *Endocrinology*, 1947, 41, 356.

⁴ Sperry, W. M., and Stojanoff, V. A., *J. Nutrition*, 1935, 9, 131.

⁵ Sayers, G., Sayers, M. A., Liang, T. Y., and Long, C. N. H., *Endocrinology*, 1945, 37, 96.

⁶ Sayers, G., Sayers, M. A., Fry, E. G., White, A., and Long, C. N. H., *Yale J. Biol. and Med.*, 1944, 10, 361.

It is well known that the adrenal cortex is an important factor in stresses not far different from this (Selye⁸). The adrenal cortical hormone is a cyclopentophenanthrene compound closely related to cholesterol and the adrenal gland is a rich source of cholesterol. There is every reason to believe that the hormone is formed from the cholesterol present in the gland. (This is true of all glandular structures which arise from embryonic lateral plate mesoderm.) Sayers *et al.*⁶ found that the adrenal cholesterol became depleted upon administration of adrenotropic hormone. Mason *et al.*⁹ have reported an increased urinary output of keto-steroids in humans after administration of adrenotropic hormone. At least one biologically active compound can be formed from cholesterol; Bloch¹⁰ fed cholesterol labeled with deuterium to pregnant women and recovered it as pregnandiol in the urine.

From the data presented here it is apparent that both total fat and cholesterol are present in lower concentration in the adrenals of wild rats than in domestic rats. Assuming the 5% figure to be the correct one for the cholesterol concentration in the adrenal of the domestic rat, the wild rat has approximately 66% as much cholesterol. However, since Rogers and Richter¹ have shown the cortex of the wild rat to be 2-3 times larger

⁷ Levine, L., *Endocrinology*, 1945, 37, 34.

⁸ Selye, H., *J. Clin. Endocrinology*, 1946, 6, 117.

⁹ Mason, H. L., Power, M. H., Bynearson, E. H., Ciaramelli, L. C., Li, C. H., and Evans, H. M., *J. Clin. Endocrinology*, 1945, 8, 1.

¹⁰ Bloch, K., *J. Biol. Chem.*, 1945, 157, 661.

TABLE II.
Titers of Positive Complement Fixation Tests in Relation to Time After Onset of Illness.

Months after onset of illness	No. positive sera	Titers							Mean positive titers
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	
12	29	0	2	3	2	11	5	6	1:34
12-23	67	0	1	6	17	24	12	7	1:30
24-35	123	4	6	28	31	38	16	0	1:18
36-47	94	1	11	15	32	19	12	4	1:18
Total	313	5	20	52	82	92	45	17	1:21

excluded from the series.

Results. In Table I, the persons on whose sera complement fixation tests were performed have been classified in groups according to the history of the presence or absence of a typical typhus rash and the presence of Weil-Felix agglutination in significantly positive or suggestive titer at the time of their illnesses. It would seem highly probable that the persons in Groups I and II had suffered from murine typhus. Weil-Felix agglutination titers of 1:320 or more are generally considered as diagnostic of rickettsial infection. Rocky Mountain spotted fever was the only rickettsial disease known to occur in Florida which might have caused the symptoms of, and the findings on, the patients in these two groups. In no instance did epidemiological evidence suggest this disease. Moreover, of the 91 sera negative for typhus, 66 were examined by the same complement fixation test for Rocky Mountain spotted fever. With the exception of 13 sera in which positive results were obtained in 1:2 dilution only, all of the sera examined for Rocky Mountain spotted fever were negative. Because of the lack of both clinical and epidemiological evidence of Rocky Mountain spotted fever, these positive results in low titer were considered non-specific.

From the data on Groups I and II presented in Table I, it would appear that the complement fixation test was approximately 85 to 88% accurate as an indicator of past infection with murine typhus for periods up to 3 or 4 years following illness. It was also apparent that positive titers were quite high. The most common titer was 1:32, and the mean was 1:21. In the individuals included in Groups III to VI, the diagnosis of typhus was not considered completely established in

every case. The possible inclusion of diseases other than typhus in these groups might account for the lower percentage of positive sera observed in the groups. This fact appeared to have no influence on the mean positive titers for Groups I and II as compared with Groups III to VI with titers of 1:21 and 1:22 respectively.

In Table II, persons with positive titers have been classified in one-year periods according to the time elapsed between illness and the taking of serum for complement fixation. The diminution in mean positive titers observed in the yearly intervals after infection was not statistically significant. This observation suggested that no pronounced loss of positive titer had occurred with the passage of time.

As a control upon the specificity of the test, the sera of 20 inmates of a New York State correctional institution were examined for murine typhus by the same technic. Of these sera, 17 were negative and 3 fixed complement in 1:2 dilution, one serum with 4 plus fixation and 2 with 3 plus. None of the 3 subjects had positive serological reaction for syphilis. Two had never been out of New York State and had never taken typhus vaccine. One, however, had had 2 years of naval service on the island of Guam ending a few months before the specimen was taken. It was, therefore, possible that this person had received typhus vaccine or may have been infected during the course of his travels. However, there had been no history of illness during the period of his absence from New York State. It would appear that at least 2, and probably all 3, of these positive reactions were nonspecific. In consideration of the higher titers commonly observed in persons who had had typhus, the occasional oc-

with murine typhus rickettsiae of the Wilmington strain. Lipoids were extracted with benzol and ether, and the antigen was purified by sodium sulfate precipitation in order to eliminate or reduce non-specific reactions with syphilitic sera.⁸

Hemolysin was titrated in 0.25 ml amounts in twofold dilutions, with starting dilutions of 1:200 and 1:300 followed by the addition of 0.25 ml of 3% washed sheep cells and 0.5 ml of guinea pig complement diluted 1:30. The highest dilution of amboceptor which showed complete hemolysis after incubation for 30 minutes at 37°C constituted one unit. This dilution divided by 3 was employed in the tests. Complement was titrated in 0.5 ml amounts in the presence of 0.25 ml of diluted antigen and 0.25 ml of normal saline. The hemolytic system of 0.5 ml of 1.5% sensitized cells was added, and results were read after incubation for 30 minutes at 37°C. Two full units of complement in 0.5 ml amounts were used in the tests. Antigen was titrated in serial twofold dilutions of 0.25 ml amounts with serial twofold dilutions of the same amounts of a positive serum. Complement was added and, after 18 hours of icebox fixation, the above-mentioned hemolytic system was added. The titration was read after incubation for 30 minutes at 38°C. The highest dilution of antigen giving 4 plus fixation with the highest dilution of serum was considered the unit of antigen. Two units in 0.25 ml amounts were used in the tests.

Serum specimens were diluted in 0.25 ml amounts in original dilutions ranging from 1:2 to 1:128. Tests were carried out in the same manner as described under titration of antigen, with the exception that the highest serum dilution in which 3 plus or greater fixation was observed was considered the end point. In tests for each day, 2 known positives and 2 known negative control sera were included, as well as a control titration of the complement with the same amount of antigen as was used in the test. Anticomplementary controls were included for all sera in the lowest dilution, and anticomplementary sera were

TABLE I.
Correlation of Clinical and Weil-Felix Agglutination Findings During Illness with Titers Observed in Late Complement Fixation Tests.

Group No.	Findings at time of illness	No. sera examined	No. positive sera	% positive	Titers									
					Complement fixation tests									
					0	1:2	1:4	1:8	1:16	1:32	1:64	1:128		
I	Rash. Weil-Felix 1:320 or more	124	109	88	15	2	5	18	34	27	19	4		
II	No rash. Weil-Felix 1:320 or more	79	67	85	12	1	6	13	14	22	7	4		
III	Rash. No serological exam.	105	77	73	28	0	5	14	12	25	14	7		
IV	Rash. Weil-Felix 1:160 only	21	14	67	7	0	1	3	4	5	1	0		
V	No rash. Weil-Felix 1:160 only	16	8	50	8	0	0	2	3	3	0	0		
VI	No rash. No serological exam.	59	38	65	21	2	3	2	15	10	4	2		
	Total	404	313	77	91	5	20	52	82	92	45	17		

⁸ Van der Scheer, J., Bohnel, E., and Cox, H. R., *J. Immunology*, 1947, 56, 365.

TABLE I. γ P/mg total N.

	Inorganic	Acid sol.	Lipid	Nucl acid
Normal brain	22.4 22.8 22.9 24.2 23.0 23.7 24.2 21.6	52.8 54.0 56.4 54.5 52.0 51.4 54.5 50.8	104.0 109.2 105.5 104.5 110.0 99.0 120.8 107.3	20.4 23.1 22.2 22.9 24.1 24.2 38.4 26.4
Encephalitic brain	26.2 23.0 26.6 22.1	57.5 52.2 52.6 49.5	108.2 108.5 107.5 107.6	21.6 22.5 25.5 29.5
Avg normal	23.1	53.3	107.5	25.2
Avg encephal.	24.5	53.0	108.0	24.8
P*	>.562	>.562	>.562	>.562
	"Protein residue"	Ribose†	Desoxyribose‡	Total
Normal brain	9.8 9.1 9.9 9.0 11.1 9.9 11.1 11.0	9.9 10.4 10.7 10.6 10.5 10.6 11.5 10.6	7.2 11.1 10.8 10.3 10.6 10.3 11.2 11.4	187.0 196.0 195.0 191.5 197.0 184.5 212.0 196.0
Encephalitic brain	10.2 11.6 10.4 11.0	8.6 10.4 8.2 9.1	11.8 11.1 10.5 10.5	198.0 195.0 196.0 189.6
Avg normal	10.1	10.6	10.4	194.9
Avg encephal.	10.8	9.0	11.0	194.6
P*	.223	.005	.389	.500

* P equals probability that the difference in means as large as that observed here would be exceeded solely through operation of chance.

† Calculated from ribose as determined on nucleic acid fraction by orcinol-HCl reaction.⁵

‡ Calculated from desoxyribose as determined on nucleic acid fraction by diphenylamine reaction.⁵

the separation of phosphorus compounds into 4 groups; namely, acid soluble, lipid, nucleic acid and "protein residue". Because of the small amounts of tissue used in the study it was felt desirable to use total nitrogen as a standard reference rather than wet weight of tissue. For this reason it is not possible to compare the data presented with those of Schneider on rat brain since in Schneider's report the total nitrogen content of the tissue was not determined.

Table I shows the values of phosphorus-containing compounds in normal and infected brain tissue.

Conclusions. Determinations of phosphorus-

us-containing compounds of the whole brain tissue taken from normal mice and from mice suffering from polio-encephalitis produced by the MM poliomyelitis virus did not reveal any significant differences in the inorganic, acid soluble, lipid, nucleic acid, "protein residue" or total phosphorus fractions. Nor did they show any significant differences in the desoxyribosenucleic acid content as determined by the diphenylamine reaction. However, the content of ribosenucleic acid as determined by the orcinol-HCl reaction was significantly reduced in infected brains as compared with normal mouse brain tissue.

currence of non-specific reaction in low dilution of serum would not seem to detract greatly from the utility of the test.

Summary. Complement fixation tests for murine typhus were performed upon the sera of 203 persons who, it is highly probable, had suffered an attack of murine typhus 7 days to 3 years and 11 months previously. The tests were positive in 85 to 88% of the cases, and in most instances the positive titers were high. Among 201 persons suspected of having had typhus, though the diagnosis was

not conclusively established, the per cent of positive reactions was less, but the titers of positive sera did not differ significantly from those of the group in which the diagnosis was considered to be established. Up to periods of 4 years after infection, mean positive titers at yearly intervals did not suggest a pronounced loss of titer with passage of time. With the exception of infrequent reactions at 1:2 dilutions in the sera of persons who had not had typhus, the test was found to be specific.

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Studies on Phosphorus-Containing Compounds in Normal and Poli-encephalitic Mouse Brain.*

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(Introduced by M. B. Visscher.)

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Chemical investigations in the field of neurophysiology have shown that all conditions leading to a depressed nerve function are associated with a breakdown of phosphocreatine.¹ In nerve cells in which chromatolysis has been produced by axon section a markedly increased acid phosphatase activity has been found. This increased activity was proportional to the degree of chromatolysis and was probably associated with increased nucleoprotein synthesis or degradation.²

In regenerating neurons which are resistant to the infection with the poliomyelitis virus a decrease of phosphocreatine could also be shown. This change together with the reduction of cytochrome oxidase and succinic dehydrogenase activity in regenerating neurons resembled the effect of cyanide on brain

metabolism.³ Changes in the acid soluble phosphorus compounds in the brain in poliomyelitis have been described, consisting in a greatly increased content of adenosine-triphosphate and a markedly decreased content of phosphocreatine and residual organic phosphate.⁴

Experiments. In the present studies phosphorus-containing compounds were determined in normal mouse brain tissue and compared with those found in the brain of animals infected with poliomyelitis. Five weeks old mice of the C₃H strain received from the colony of Dr. John J. Bittner, University of Minnesota, were inoculated intraperitoneally with the MM poliomyelitis virus. At the height of the infection the animals were decapitated, the brain rapidly removed and homogenized in cold 5% trichloroacetic acid. Phosphorus compounds were determined by the method of Schneider⁵ which allows for

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¹ Gerard, R. W., and Waller, J., *Am. J. Physiol.*, 1929, 89, 108.

² Bodian, D., and Mellors, R. C., *Proc. Soc. Exp. Biol. and Med.*, 1944, 55, 243.

³ Bodian, D., and Mellors, R. C., *J. Biol. Chem.*, 1947, 167, 655.

⁴ Kabat, H., *Science*, 1944, 90, 63.

⁵ Schneider, W. C., *J. Biol. Chem.*, 1945, 161, 293.

TABLE I.
Fertility in Relation to Number of Spermatozoa.

No. sperm. per insem. × 1000	Dilution rate	Vaginal group			Uterine group		
		No. eggs	% fert.	Avg % fert.	No. eggs	% fert.	Avg % fert.
92	1/1000	10	100	100	8	100	100
		7	100		0	—	
61	1/1000	7	71	36	5	0	0
		5	0		6	0	
46	1/1000	11	100	100	7	100	100
		8	100		0	—	
32	1/2500	8	50	50	10	70	35
		0	—		10	0	
24	1/2000	13	100	81	12	100	100
		8	62		5	100	
17	1/3500	9	0	0	5	0	0
		9	0		9	0	
17	1/4500	9	0	0	8	0	14
		16	0		11	27	
16	1/3500	9	11	20	12	0	15
		10	30		10	30	
16	1/5000	8	62	62	6	0	0
		0	—		7	0	
Group fertility (unweighted avg)				50			40

therefore, received approximately equal amounts of suspension containing approximately equal numbers of spermatozoa.

The insemination was made about 1½ to 3½ hours after the sterile mating (only sperm free seminal fluid deposited in vagina by the vasectomized male). Autopsy was made 25 to 30 hours after insemination; the oviducts were flushed, and fertilization of the ova was determined microscopically from the presence of 2 to 8 equal blastomeres.

Results. The findings of the present study show that partial fertility can result from a very small number of spermatozoa per insemination, as few as 16,000 (Table I). This was true in both vaginal and uterine inseminations. Maximum fertility was obtained with a number of spermatozoa around 90,000, and fertility was affected greatly when the number was cut below 20,000. A negative relationship was found between the dilution rates and the fertilizing capacity of the spermatozoa but the effect of a small number of sperm was confounded with the effect of high dilution. The effects of dilution rate upon motility were observed incidentally but the effect of this factor upon fertility also was confounded with number and concentration. The average fertility resulting from vaginal insemination was higher than that

from uterine insemination (50% vs 40%). However, statistical analysis of the data from the 2 groups failed to show a significant difference between them ("t" test: $P > .05$). If the vaginal group could have been inseminated at laparotomy similar to the uterine group, the data could be considered more critical in comparing the effects of the two placements upon fertility.

Experiment II. Motility of spermatozoa as affected by high dilutions with different diluents. The above data show that the number of spermatozoa necessary either for maximum or for partial fertility is less than that previously reported. The discrepancy between different results may be due to the difference in diluents. The effects of high dilution upon motility of spermatozoa in a series of dilutions with both 0.9% sodium chloride and Krebs solutions were investigated next.

Material and methods. Twelve semen samples from 9 males were collected at different times and diluted in 8 successive dilutions with both 0.9% sodium chloride and Krebs solutions from 1:100 to 1:12800. Semen was introduced into containers of adequate sizes by a 0.01 cc graduated pipette; the diluent was then added slowly, and shaken gently in order to obtain an even dispersion

Fertility in Rabbit as Affected by the Dilution of Semen and the Number of Spermatozoa.*

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Walton¹ first demonstrated in the rabbit that fertility is influenced by the number of spermatozoa introduced into the vagina; maximum fertility was obtained when the number of spermatozoa was more than 1,000,000. The semen was diluted in 0.9% sodium chloride solution and 3 cc of semen suspension was used per insemination. Rowlands² used Baker's solution to dilute the semen and 2 cc of such semen suspension was deposited per insemination. He found the same number of spermatozoa necessary for maximum fertility as Walton did and sterility was reached when the number was dropped to 100,000. Chang³ also used 0.9% sodium chloride solution as the diluent of rabbit semen with only 1 cc per insemination, and obtained a much lower figure on the number of spermatozoa for both maximum and minimum fertility (for maximum fertility: 330,000-420,000; for minimum fertility: 40,000). Chang⁴ also tested the effect of dilution on the fertilizing capacity of rabbit sperm by inseminating the same number of spermatozoa suspended in various volumes of physiological saline (1.0 cc, 0.4 cc and 0.1 cc). The advantage appeared to be with the least volume.

The present studies attempt to determine the minimal number of sperm for fertility from insemination into the vagina and into the uterine horns with highly diluted semen. Further, an attempt was made to determine the effects of high dilution with physiological

saline and Krebs solutions upon the motility of spermatozoa. Also the effect of high dilution with Krebs, and its consequent effect upon motility, upon the fertilizing capacity of the sperm was determined.

Experiment I. Fertility in relation to number of spermatozoa.

Material and methods. Thirty-six female rabbits were used in this study. All animals were in heat and mated to vasectomized bucks to induce ovulation. Semen samples were collected from 2 or 3 normal males with an artificial vagina⁵ and pooled together. The number of spermatozoa was determined from the average counts of 2 independent hemocytometer samples. Four hundred hemocytometer squares were counted in each case and no pairs of counts were accepted which showed differences of statistical significance between them. The initial motility of spermatozoa was estimated in ranks or grades. The semen was then diluted with Krebs solution, modified according to Lardy and Phillips⁶ until the desired number of spermatozoa per unit of volume was obtained and then kept in a warm water bath at approximately 37°C for the short time until it was used.

Four females were used in each trial with 2 in each group. In the "vaginal group" 0.2 cc of semen suspension was deposited into the anterior portion of the vagina by an inseminating tube; in the "uterine group," a laparotomy was made under light anesthesia and 0.1 cc of the same semen suspension was injected into the tubal ends of each of the uterine horns with a ¼ cc tuberculin hypodermic syringe. Animals in both groups,

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² Rowlands, I. W., *Nature*, 1944, 154, 332.

³ Chang, M. C., (In "The Problem of Fertility," 1946. Edited by E. T. Engle, Princeton Univ. Press, p. 169), 1946.

⁴ Chang, M. C., *Science*, 1946, 104, 361.

⁵ Macirone, C., and Walton A., *J. Agr. Sci.*, 1938, 28, 122.

⁶ Lardy, H. A., and Phillips, P. H., *Amer. J. Physiol.*, 1942, 138, 741.

TABLE IV.
 Fertility of Rabbits in Relation to Dilution and Motility of Spermatozoa.

Motility rank*	200,000/cc 0.1 cc/insem.		100,000/cc 0.2 cc/insem.		50,000/cc 0.4 cc/insem.		25,000/cc 0.8 cc/insem.		Total No. eggs	Avg % fertility
	No. eggs	% fert.	No. eggs	% fert.	No. eggs	% fert.	No. eggs	% fert.		
1					6	0	8 11 7	0 91 0	32	22.7
2					8 7	50 0	10	100	25	50.0
3			7 8 9	100 25 0	6	100	5	0	35	45.0
4	9 11	100 0	8	38	11 9	73 0			48	42.2
5	7 9 8	100 0 100	7 8	100 100					39	80.0
Total No. eggs	44		47		47		41		179	
Avg % fertility		60.0		60.5		37.2		38.2		

* 1, no motion at all; 2, oscillatory motion; 3, motility from 1% to 25%; 4, motility from 25% to 50%; and 5, motility from 50% to 75%.

different motility groups, however, showed a range of 57.3% from 22.7 for grade 1 motility up to 80.0 for grade 5.

A significant correlation was found between motility and fertility, 0.43, but an insignificant correlation was shown between sperm concentration and fertility, 0.22. The motility of the spermatozoa and the sperm concentration showed a rather high correlation, 0.78. The partial correlation between fertility and motility with concentration held constant was 0.42 whereas between fertility and concentration with motility held constant it was -0.17 and insignificant. Under the conditions of high dilutions, the motility of the spermatozoa appears to have some value for predicting fertility. It also appears that much of the deleterious effect of high dilution upon fertility of sperm is brought about through its inhibitory action upon motility.

Summary. Female rabbits were inseminated with semen diluted with Krebs solution at rates of 1/1000 up to 1/5000 and 0.2 cc of suspension were used. The number of

spermatozoa effective for partial fertility was 16,000, and for maximum fertility was around 90,000. The group percentage of fertility resulting from vaginal insemination was slightly higher than from uterine insemination (50% vs 40%); this difference was not of statistical significance.

When semen samples were diluted from 1:100 up to 1:12800, the motility of the spermatozoa decreased significantly as the rates of dilution increased and the average motility shown in Krebs solution and the maximal dilution that would support motility was significantly higher than in 0.9% sodium chloride solution.

When females were inseminated with a small number of spermatozoa (20,000) suspended in different volumes of Krebs solution, the average percentages of fertility were higher in the smaller volumes. The motility of the spermatozoa, however, appeared to have a greater effect on the fertility than the dilution.

TABLE II.
Motility and Highest Dilution Showing Motility of Rabbit Spermatozoa in Two Diluents.

Sample No.	Avg % motility in 8 dilutions (from 1:100 to 1:12800)		Highest dilution showing motility	
	0.9% NaCl	Krebs	0.9% NaCl	Krebs
1	29.4	36.2	3200	12800
2	21.2	36.2	800	3200
3	13.1	24.1	400	12800
4	6.4	20.6	400	6400
5	19.4	32.0	800	6400
6	6.3	10.4	200	3200
7	13.4	20.6	800	3200
8	1.4	5.1	200	400
9	30.1	38.7	3200	6400
10	8.1	13.9	400	1600
11	23.1	43.9	1600	3200
12	0.9	6.9	400	800
Avg	14.4	24.1	1033	5033

of spermatozoa. All semen suspensions were kept in the warm water bath for 30 minutes and the percentage of spermatozoa showing progressive motion then estimated.

Results. Almost without exception, the average motility in the 8 dilutions and also the highest dilution rate supporting motility was higher for Krebs solution than for the 0.9% sodium chloride solution (Table II), and a trend was shown in both diluents that

TABLE III.
Progressive Motility of Rabbit Spermatozoa in Dilutions from 1:100 to 1:12800.

Dilution	Avg percentage motility in	
	0.9% NaCl	Krebs
1: 100	48.3	57.9
1: 200	37.7	47.5
1: 400	18.2	35.5
1: 800	8.0	23.8
1: 1600	2.5	17.4
1: 3200	0.5	8.6
1: 6400	0	1.2
1:12800	0	0.6
Avg	14.4	24.1

the higher the dilution rate the lower the motility (Table III). Statistical analyses showed that the motility of spermatozoa varied significantly from one dilution to another, and the average difference between the diluters was also highly significant ("F" test, $P < 0.01$).

Experiment III. Fertility as affected by rates of dilution and motility of spermatozoa.

The low motility observed in the high dilutions causes one to recall the results in Experiment I, in which the rates of dilution were from 1/1000 up to 1/5000. Considering the fact that the average highest dilution showing motility was 1:1033 in 0.9% sodium chloride solution and 1:5033 in Krebs solution, it was suspected that the motility of the spermatozoa is one of the determining factors in fertilization and the difference in solutions accounts for the discrepancy between present results and those of former workers. The effect of dilution upon the fertilizing capacity of the spermatozoa was therefore studied next.

Material and methods. The same small number of spermatozoa, viz., 20,000 all from the same sample of semen, were suspended in 0.1 cc, 0.2 cc, 0.4 cc and 0.8 cc of Krebs solution. Four animals were used for each trial, each animal received a different volume of suspension; altogether there were 7 trials in this study. The same procedure as mentioned in Experiment I was followed except the semen suspension was deposited in the vagina in all animals. The motility of spermatozoa was estimated on each semen suspension before the insemination.

Results. The fertility data are based on 22 animals; the remaining 6 failed to ovulate. The average fertility in each of the 4 different volume groups was: 0.1 cc, 60.0%; 0.2 cc, 60.5%; 0.4 cc, 37.2%, and 0.8 cc, 38.2% (Table IV) for a range of 23.3%. The

although natural and synthetic nucleosides are beta-glycosides, the sugars differ in their ring structures. For these reasons the synthetic nucleosides might be expected to show microbiological properties different from uridine and from the uracil and thymine derivatives.

Results obtained in testing these synthetic nucleosides as possible metabolite antagonists for various microorganisms are reported in this paper.

Tests with Escherichia coli. Two strains of *E. coli* were used: a uracil-requiring mutant strain (550-460)[†] and a strain (American Type Culture Collection No. 9723) which synthesizes its own uracil. Good growth of the uracil mutant was obtained when uracil (0.005 to 0.04 mg per 7.5 ml) or natural uridine[§] (0.01 to 0.1 mg per 7.5 ml) were added to the medium described by MacLeod.¹² The tests with this mutant were carried out in the presence of 0.015 or 0.02 mg of uracil per 7.5 ml of medium. These levels of uracil permitted about 50% of normal growth. When the uracil and thymine derivatives were tested in concentrations up to 1 mg per 7.5 ml of medium, only 5-chlorouracil was slightly stimulatory, while none of the following showed any growth-promoting or growth-inhibiting properties: 1-D-ribosyluracil, 1-D-arabinosyluracil, 1-D-glucosyluracil, 1-D-galactosyluracil, 1-D-ribosyl-5-chlorouracil, 1-D-arabinosyl-5-chlorouracil, 1-D-glucosyl-5-chlorouracil, 1-D-galactosyl-5-chlorouracil, 1-D-ribosyl-5-bromouracil, 1-D-arabinosyl-5-bromouracil, 1-D-glucosyl-5-bromouracil, 1-D-galactosyl-5-bromouracil, thymine, 1-D-ribosylthymine, 1-D-arabinosylthymine, 1-D-glucosylthymine, and 1-D-galactosylthymine. None of these compounds, when tested in concentrations up to 20 mg per 7.5 ml of medium, inhibited the growth of the American

Type Culture Collection No. 9723 strain of *E. coli*.

Tests with Neurospora crassa.^{||} The wild strain of *Neurospora crassa*, Abbott 4A was grown in the medium described by Horowitz and Beadle.¹³ With the uracil-less mutant, *Neurospora crassa*, 1298, a typical growth curve was obtained when either uracil or natural uridine[§] was added to the medium. To obtain high enough concentrations without using too much of the various compounds, all tests with *Neurospora* were carried out in 10 ml of medium. In this amount of medium maximum growth was equivalent to 20 to 30 mg of dried mycelia. All tests with the uracil-less mutant were carried out in the presence of 0.1 mg uridine in 10 ml of medium. Of all the compounds listed in the previous section, when tested in concentrations up to 0.8 mg per 10 ml of medium, only 5-chlorouracil slightly stimulated the growth of the uracil-less mutant. None of the compounds in concentrations up to 20 mg per 10 ml inhibited the growth of either the wild or uracil-less strains of *Neurospora*.

Tests with Lactobacillus casei. All tests with *L. casei* (American Type Culture Collection, No. 7469) were carried out in the medium and according to the procedure described by Luckey, Briggs, and Elvehjem.¹⁴ In the absence of added pteroylglutamic acid the growth reached a level of 20 turbidity units, whereas the addition of 0.010 mg of thymine permitted maximum growth equivalent to 130 turbidity units. The following compounds consistently stimulated the growth of *L. casei*: (The percentage values given in the parentheses indicate the relative molar activity as compared with thymine taken as 100%): 1-D-ribosylthymine (1.0%), 1-D-glucosylthymine (1.4%), 1-D-galactosylthymine (0.5%), and 1-D-arabinosyl-5-chlorouracil (0.3%). In the presence of 0.002 mg of thymine per 10 ml medium the follow-

[†] The cultures of *E. coli*, American Type Culture Collection No. 9723 and the uracil mutant, *E. coli*, (550-460), were obtained through the courtesy of Dr. J. O. Lampen of the American Cyanamid Company of Stamford, Conn.

[§] The authors are indebted to Dr. Hubert Loring of Stanford University for a generous sample of natural uridine.

¹² MacLeod, C. M., *J. Exp. Med.*, 1940, **72**, 217.

^{||} The authors wish to thank Dr. Herschel Mitchell and Dr. Hubert Loring for kindly supplying cultures of *Neurospora*.

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¹⁴ Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

Some Microbiological Properties of Synthetic Nucleosides.*

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Increasing interest in the roles of pyrimidines, purines, nucleosides, and other nucleic acid derivatives in the living cell has directed the attention of many investigators to various biological systems which utilize these compounds. Through X-ray irradiation, Beadle and co-workers¹ obtained mutants of *Neurospora crassa* which required added uracil or uridine for growth. Likewise, mutants of *Escherichia coli* which require uracil for normal growth have been developed and studied.²

The *L. casei* factor (pteroylglutamic acid) requirements for the growth of *Lactobacillus casei*, *Streptococcus faecalis* R, and certain other microorganisms can be fulfilled by thymine.³

Because specific metabolite antagonists have been useful in determining some of the biological functions of metabolites as well as changing the growth characteristics of living cells, we synthesized a number of derivatives of uracil⁴ and thymine⁵ as possible antagonists of these metabolites. The possibility that these derivatives might enhance the activity of the corresponding natural metabolite was equally interesting.

Emerson and co-workers⁶ found that a specific antagonist of riboflavin was obtained when the ribityl group was replaced by a dulcetyl group. This suggested the possibility of producing an antagonist of uridine by substituting galactose or other sugars for ribose of the nucleoside molecule.

Kuhn and co-workers⁷ reported the 6,7-dichloro derivative of riboflavin as a specific antagonist of riboflavin. Hitchings, Falco and Sherwood⁸ found that 5-chlorouracil and 5-bromouracil were antagonists of thymine. In both of these instances the substitution of a chlorine atom for a methyl group produced an antagonist. Since Loring and Pierce⁹ found natural uridine from 10 to 60 times more effective than uracil as a growth promoter for a uracil-less mutant of *Neurospora*, it seemed possible that the ribosyl and other glycosyl derivatives of 5-chloro- and 5-bromouracil might be more effective thymine antagonists than 5-chlorouracil or 5-bromouracil.

To test these various possibilities, the ribosyl, arabinosyl, glucosyl, and galactosyl nucleosides of uracil¹ and thymine⁵ were synthesized, as well as the corresponding 5-chloro- and 5-bromouracil derivatives. Although synthetic 1-D-ribosyluracil is chemically similar to naturally occurring uridine it has different physical properties.¹⁰ Recent work of Todd and co-workers¹¹ suggests that,

* This work was started with the aid of a University of Colorado Research Grant and continued in part by a research contract with the Office of Naval Research.

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Effects of Dehydrating Agents on Phosphatases in the Lymphatic Nodules of the Rabbit Appendix.

WILLIAM L. DOYLE.*

From the Department of Anatomy, University of Chicago.

In the course of a study on the quantitative correlation between phosphatase activities and histological changes in lymphatic tissue it has been necessary to determine storage properties and stability to dehydration of these enzymes in our material. The rabbit appendix was selected because it presents an almost diagrammatically homogeneous distribution of lymphatic nodules over a large area. This arrangement is ideal for histochemical examination of the effects of experimental modifications.¹ Rabbits were killed with nembutal and the appendix promptly removed, washed with saline, drained, weighed and dehydrated as indicated or minced and homogenized fresh. Strips weighing about one gram each were made up to 20 ml of homogenate. Determinations were carried out in triplicate at 30°C and 40°C with 2.5-5.0 mg of fresh weight of tissue in suspension. Disodium phenylphosphate (0.5%) was used as substrate with barbital or glycine buffers. The reaction was stopped at the end of 30 minutes by addition of Folin-Ciocalteu phenol reagent. No activation was found with added magnesium. Tissues to be dehydrated were dropped into 50 volumes of absolute acetone, 80% acetone, absolute alcohol, 80% alcohol, absolute methyl alcohol (all at room temperature) or into isopentane chilled in liquid nitrogen. The samples frozen in isopentane were dehydrated at -40°C *in vacuo*. The other samples were left in the fixative at 5°C for 24 hours and then drained and homogenized in water in a Potter-Elvehjem homogenizer.

Alkaline phosphatase. The optimum for alkaline phosphatase activity was found to

be at pH 9.8 with 20% less activity at 9.6 and 10.1. Freshly homogenized appendix gave values for 6 different rabbits of 7.0 to 11.0 μg phenol liberated per milligram fresh weight of tissue in 30 minutes at 30°C. The activity at 40°C was 1.35 times as great. Single values in the triplicate determinations at each step agreed within 1%. Taking the fresh homogenate as 100% activity we found in the different specimens $95 \pm 5\%$ activity after storage of the homogenate at 5°C for 24, 48, 72 and 96 hours. The frozen dried tissue gave $85 \pm 8\%$ of the fresh value at periods of 1, 48 and 96 hours after homogenizing. After fixation in absolute methyl alcohol only 15% activity remained. When fixed for 24 hours in acetone or alcohol and then homogenized, the immediate values were as follows: absolute acetone, 80% acetone and absolute alcohol gave $75 \pm 5\%$ of the fresh activity; 80% alcohol gave $65 \pm 5\%$ activity. The acetone or alcohol fixed enzyme preparations underwent further inactivation upon storage as homogenates at 5°C at comparable steady rates so that 72 hours later about two-thirds of the initial values after fixation were obtained. Each of these fixed samples carries about 3% of the fixative into the homogenate suspension. In this material 80% alcohol has always preserved less alkaline phosphatase activity than either absolute alcohol or acetone which is at variance with the results of Stafford and Atkinson.² The frozen dried material has only slightly higher activity than the absolute alcohol and acetone fixed material but it is as stable upon storage as are fresh tissue homogenates. Frozen dried material stored dry in a desiccator at 25°C for 3 days showed a decrease of activity to about 30% but retained full activity for two weeks when stored at -20°C.

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Tests with Streptococcus faecalis R. The tests with *Strep. faecalis R*[†] (American Type Culture Collection, No. 8043) were made in the medium and according to the procedure referred to above.¹⁴ The results obtained were almost identical with those obtained with *L. casei*. The compounds which showed some stimulation in the absence of thymine were: 1-D-ribosylthymine (2.2%), 1-D-galactosylthymine (0.7%), and 1-D-glucosylthymine (1.1%). The following compounds were slightly stimulatory in the presence of 0.002 mg of thymine per 10 ml of medium but were inactive alone: 1-D-ribosyl-5-chlorouracil, 1-D-arabinosyl-5-chlorouracil, 1-D-glucosyl-5-chlorouracil, 1-D-galactosyl-5-chlorouracil, 1-

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At a concentration of 0.020 mg per 10 ml of medium 5-chlorouracil inhibited the growth to 100% in the presence of 0.002 mg of thymine. None of the other compounds up to a concentration of 1.0 mg per 10 ml showed any growth-inhibitory properties. However, when the concentration of these compounds was greatly increased, some of them were toxic. Galactosyluracil and 5-chlorogalactosyluracil, at levels of 10 and 5 mg, respectively, inhibited the growth to 50% of that obtained with 0.002 mg of thymine.

Summary. A number of synthetic nucleosides, and chloro- and bromo- derivatives of these nucleosides, were tested for growth-promoting and growth-inhibiting properties on *Escherichia coli*, strain 9723, and its uracil-requiring mutant, *E. coli*, strain 550-460; *Neurospora crassa*, wild strain Abbott 4A, and a uracil-less strain of *Neurospora crassa*, No. 1298; and on the growth of *Lactobacillus casei* and *Streptococcus faecalis R*.

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Problems in Determining the Bacterial Flora of the Pharynx.*

COMMISSION ON ACUTE RESPIRATORY DISEASES.†

(In collaboration with Walter A. Mickle and Thomas J. Oliver.)

From the Respiratory Diseases Commission Laboratory, Regional Station Hospital, Section 2,
Fort Bragg, N. C.‡

The role of bacteria in the pathogenesis of the common respiratory diseases has not been clearly defined. Attempts have been made to correlate the pharyngeal flora with the presence or absence of upper respiratory infection and to determine whether or not changes in flora occur during the course of illness.¹⁻¹⁴ The results have been inconstant

and difficult to interpret. Consideration of the methods employed suggests that technical and nutritional limitations may have been responsible, in some instances, for apparent differences in flora, while in other instances, such limitations may have obscured changes in flora that were present.¹² A study has therefore been made to define some of the problems of interpreting the results of pharyngeal cultures and to develop a technic that would yield reproducible as well as interpretable data. The investigations were carried out in conjunction with other studies on respiratory disease.¹⁵⁻¹⁸

* This investigation was supported through the Commission on Acute Respiratory Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Office of the Surgeon General, U. S. Army, and by grants from the Commonwealth Fund, the W. K. Kellogg Foundation, the John and Mary R. Markle Foundation and the International Health Division of the Rockefeller Foundation to the Board for the Investigation and Control of Influenza and Other Epidemic Diseases for the Commission on Acute Respiratory Diseases.

† Members and professional associates of the Commission on Acute Respiratory Diseases were: John H. Dingle, Director; Theodore J. Abernethy; George F. Badger; Joseph W. Beard; Norman L. Cressy; A. E. Feller; Irving Gordon; Alexander D. Langmuir; Charles H. Rammekamp, Jr.; Elias Strauss; Hugh Tatlock.

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¹ Bloomfield, A. L., *Johns Hopkins Hosp. Bull.*, 1921, **32**, 33.

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This report presents certain of the results under 3 headings: a) technic, b) quantitative growth and nutritional interdependence, and c) use of a selective medium for detecting beta-hemolytic streptococci.

Technic. Two of the technical difficulties

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¹⁵ Commission on Acute Respiratory Diseases, *J.A.M.A.*, 1944, **125**, 1163.

¹⁶ Commission on Acute Respiratory Diseases, *Am. J. Pub. Health*, 1945, **35**, 675.

¹⁷ Commission on Acute Respiratory Diseases, *Johns Hopkins Hosp. Bull.*, 1946, **79**, 97.

¹⁸ Commission on Acute Respiratory Diseases, *Medicine*, 1947, **26**, 465.

Acid phosphatase. Maximal acid phosphatase activity was obtained at pH 5.6 - 5.8 with about 10% less activity at 5.4 and 6.2. The freshly homogenized tissues gave individual values for the same 6 rabbits ranging from 10 to 14 μ g phenol liberated per mg fresh weight of tissue in 30 minutes at 30°C. Taking the value one hour after homogenizing as 100% activity we found upon storage at 5°C: 75% activity after 24 hours, 70% after 48 hours, and 55% after 96 hours. Acetone (80% or 100%) preserved 25% of the fresh activity and upon storage of the homogenate for 72 hours this decreased to 15%. Alcohol (80% or 100%) preserved 15% of the fresh activity and upon storage as homogenate this declined rapidly (24 hours) to 2%. Methyl alcohol left negligible activity. The frozen dried tissue gave 85% of the fresh activity and after 48 hours had 70% of the initial fresh value which was the same as that of the fresh material after storage for the same period. Frozen dried material stored dry in a desiccator at 25°C for 3 days showed a decrease of activity to about 15% but was stable at -20°C.

Effect of temperature of fixation. In a subsequent series of experiments with 4 rabbits it was found that for acetone fixed material distinctly better cytological fixation and better preservation of both alkaline and acid phosphatase was obtained when tissues were dropped into acetone at -20°C and stored at this temperature for 48 hours.

Effect of embedding in paraffin. A sample of acetone fixed material with an activity of 9.3 μ g phenol per mg fresh weight at pH 5.6 and 8.4 μ g phenol per mg fresh weight at pH 9.8 was transferred to benzene and then to paraffin, at 60°C for 1 hour. A paraffin

block was made and stored 24 hours at 5°C. Most of the paraffin was cut away and the small block extracted with carbon disulfide at 40°C in a Soxhlet apparatus. When homogenized in water this material showed 60% of the acid phosphatase activity of the original acetone fixed material and 55% of the alkaline phosphatase activity. Analyses of sections in which the weight of the section was estimated from its calculated volume is consistent with these findings. In the work on sections it was found that the phosphatases were stable in the paraffin block even at room temperature for several days but that sections in paraffin ribbons deteriorated at room temperature although they were stable for a week at -20°C.

Summary and Conclusions. Alkaline phosphatase in the rabbit appendix is almost as well preserved by fixation in chilled acetone as by the freezing drying technic. Acid phosphatase on the other hand is distinctly better preserved by dehydration in the frozen state than after acetone fixation. Acid phosphatase activities are maximal when determined promptly on fresh homogenates. Frozen tissues dehydrated in the frozen state and stored dry at -20°C gave acid phosphatase activities equal to 85% of fresh values and for homogenates stored at 5°C for 24-96 hours the values of fresh and frozen preparations were identical. Acetone and alcohol fixation gave marked reduction in acid phosphatase activity. Alkaline phosphatase activity of fresh tissue is reduced to about 75% of the fresh value by treatment with acetone or absolute alcohol at room temperature. The effects of temperature of fixation, storage in the dry state and embedding in paraffin are noted.

TABLE II.
Relative Frequency of Various Organisms in a Series of Experiments in Which Five Plates
Were Prepared from Each Swab.

Swab No.	Readings* Plate No.				
	1	2	3	4	5
<i>Beta-hemolytic streptococci</i> (a)					
1	0	0	0	0	1
9	0	0	0	0	1
15	+	+	+	+	+
20	5	3	1	3	0
22	6	13	3	3	6
30	+	+	+	+	+
33	+	+	+	+	+
<i>H. influenzae</i> (a)					
4	NR	0	15	15	0
5	7	0	0	5	0
8	4	0	13	0	0
12	1	0	0	0	0
13	2	0	0	3	3
14	6	4	0	2	2
15	3	0	5	1	2
16	2	3	0	0	0
18	0	1	0	7	1
21	+	+	+	+	+
22	0	1	1	0	0
23	11	6	12	7	9
24	0	1	0	0	2
28	+	+	+	+	+
29	10	14	15	9	18
30	0	0	3	0	2
31	++++	++++	++++	++++	++++
32	10	5	8	2	14
34	+++	+++	+++	+++	+++
35	+	+	+	+	+
<i>S. aureus</i> (b)					
1	0	11	0	2	2
5	1	0	0	0	0
8	0	0	0	0	+
11	2	1	0	0	0
17	0	8	0	0	0
18	0	0	0	0	1
20	0	0	3	0	0
<i>H. hemolyticus</i> (c)					
11	0	1	0	4	0
12	0	3	0	0	3
13	3	7	12	11	8
14	0	0	1	0	0
16	5	1	3	6	2
19	0	2	0	0	0
20	3	2	4	0	1

* NR = Not Read. Numerals indicate number of colonies of the organisms, which were counted if less than 20.

+
 +++
 ++++

If more than 20 colonies of the organisms; this scale is an estimate of the relative number of colonies of this organism to the total number of colonies on the plate.

(a), (b), (c); plates examined for these organisms were from individuals numbered: (a) 1-35; (b) 1-20; (c) 11-20.

suspending organisms would be one resulting in a random distribution of colony-producing units (whether they be single or clumped organisms) within the resulting suspension. The distribution of such units in a suspension

may be determined by making plate counts on measured aliquots from the suspension. The total variation in plate counts should arise from a combination of technical errors or limitations, and sampling variation.

TABLE I.
Comparison of Results with Two Methods* of Examining Throat Swabs.

Organism	Dry swab positive Suspension positive	Dry swab positive Suspension negative	Dry swab negative Suspension positive	Dry swab negative Suspension negative
	No. of swabs			
<i>Beta-hemolytic streptococci</i>	2	1	4	69
<i>S. aureus</i>	1	0	3	72
<i>H. influenzae</i>	51	0	11	14
<i>H. hemolyticus</i>	52	3	4	17
Total	106	4	22	172

* See text.

in culturing the throat reside in (1) the transference of bacteria from the cotton swab to the culture plate in a manner such that the resultant growth will reflect quantitatively the various species of bacteria on the swab, and (2) the visual identification of species by their colonial morphology prior to picking the colony and identifying the pure culture so obtained.

The problem of transferring bacteria from the swab to the medium was approached by comparing the results obtained by streaking the swab directly on the medium in the manner previously employed,¹² with those obtained by suspending the material on the swab in broth prior to inoculation of the medium.

Each of 76 swabs, rubbed thoroughly over the tonsils and oropharynx, was streaked directly onto a blood agar plate. Each swab was then immersed in 5 ml of tryptose-phosphate broth. With the swab in place, the broth was drawn into and expelled from a 5 ml pipette 20 times. From this tube 0.5 ml was then transferred to a second 5 ml portion of broth, and mixed as above with the same pipette. Using a 1.0 ml pipette, 0.1 ml of the suspension was transferred to the surface of a blood agar plate (tryptose-phosphate agar base). The inoculum was spread with a glass rod which had been immersed in 95% alcohol and flamed. Spreading was facilitated by revolving the plate on a turntable. After incubation at 37°C for 24 hours the plates inoculated by each method were examined with a wide field binocular microscope ("colony" microscope). Typical colonies were picked and identified by standard confirmatory procedures.

Table I shows the results obtained in respect to 4 bacterial species. In 22 instances one or more of these 4 species was detected by the broth suspension method and not by the dry swab method, while the reverse situation obtained in only 4 instances. These data indicate that the bacterial contents of a swab can be determined better by preparing a suspension in broth than by direct inoculation from the swab.

Further experiments were designed to determine the variations encountered in the broth suspensions. In each experiment, a throat swab was obtained as described above and a broth suspension prepared. Each of 5 plates was then inoculated with 0.1 ml of the suspension. After incubation, the plates were examined with a "colony" microscope and representative colonies picked and identified. The plates were given code numbers so that the examiner was not aware of which plates were inoculated from each suspension. The results of 35 experiments are shown in Table II.

The variations encountered were greater than should arise from a homogeneous suspension, but were less than those observed in similar earlier experiments where mixing was known to have been inadequate. It is apparent that certain organisms were recovered from many of the suspensions with such low frequency that they would have been missed on a single plate regardless of the method of mixing employed.

In order further to evaluate the degree of homogeneity that could be achieved in a bacterial suspension, experiments were carried out with pure cultures. A satisfactory method of

TABLE III.

Accuracy of Identification of Colonies with a) No Visual Aid and b) Using the Microscope.

Colony tentatively identified as	(a) No visual aid		(b) Using microscope	
	No. of colonies	% confirmed	No. of colonies	% confirmed
<i>Beta</i> -hemolytic streptococcus	75	40	182	92
<i>S. aureus</i>	20	95	142	96
Pneumococcus	66	5	337	51
<i>H. influenzae</i>	174	53	566	99
<i>H. hemolyticus</i>	94	82	486	97

TABLE IV.

Distribution of Fields of 100 Colonies of a) a Mixture of *Beta*-hemolytic Streptococci and *S. aureus*, and b) a Mixture of *Beta*-hemolytic Streptococci and *H. influenzae*, According to Number of Streptococci per Field.

Deviation* from mean number of Strep. per field	a) Mixture of <i>beta</i> -hemolytic streptococci and <i>S. aureus</i>		b) Mixture of <i>beta</i> -hemolytic streptococci and <i>H. influenzae</i>	
	No. of fields		No. of fields	
	Observed	Expected	Observed	Expected
-2.0 or more		3.3		1.2
-1.5 to -1.9	1	6.4		2.2
-1.0 to -1.4	12	13.3		4.6
-0.5 to -0.9	28	21.7	8	7.5
0 to -0.4	25	27.8	19	9.6
0 to 0.4	42	27.8	17	9.6
0.5 to 0.9	24	21.7	6	7.5
1.0 to 1.4	12	13.3		4.6
1.5 to 1.9	1	6.4		2.2
2 or more		3.3		1.2
Total	145	145	50	50

* In units of \sqrt{npq}

other bacteria, including *Streptococcus hemolyticus*.¹⁹

In the first group of experiments a 3 ml broth suspension of *S. aureus* and *beta*-hemolytic streptococci was mixed with a 5 ml pipette 20 times. One-tenth ml of this mixture was placed in the center of each of 5 blood agar plates. Each plate was then rotated, using a mechanical turntable, while a sterile glass spreader distributed the inoculum evenly. After incubation at 37°C for 24 hours, the plates were examined with a wide field binocular microscope.

In order to learn whether or not the two organisms were distributed at random over the surfaces of the plates, colonies were counted differentially in fields of 100 colonies each. Forty-five to 50 such fields were counted on the 5 plates. This experiment was repeated 3 times, for a total of 145 fields.

If the colonies of streptococci and staphylococci were distributed at random, the distribution of the number of streptococci per field should be a normal one with a standard deviation of \sqrt{npq} (where p is the proportion of streptococcal colonies, q the proportion of staphylococcal colonies, and n , the number of colonies per field ≈ 100).

Table IV summarizes the differential counts of 145 fields in terms of deviation from the mean^{||} and shows that less variation was observed than expected. For example, the expected number of fields with a deviation

^{||} For example, in 50 fields counted from one series of 5 plates the average number of streptococci per field was 24.6, and the standard deviation was 4.3 streptococci per field. Eight of these fields showed 27 colonies of streptococci; this was 2.4 colonies more than the average. On a scale of standard deviations these 8 observations fall at $2.4/4.3 = +.56$ standard deviation from the mean.

¹⁹ Davis, D. J., *J. Infect. Dis.*, 1921, 29, 178.

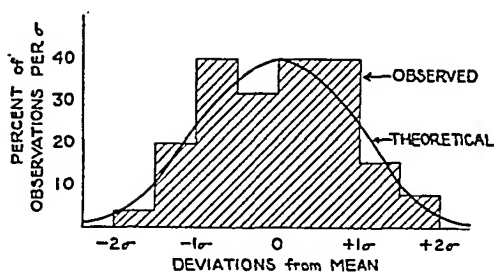


FIG. 1.

Variation in plate counts in a series of experiments in each of which several plates were inoculated with portions of a given suspension.

A loopful of broth culture of *beta*-hemolytic streptococci was transferred to 10 ml of broth and shaken a few times by hand. One loopful was then transferred to 3 ml of broth. The inoculated broth was mixed 20 times with a 5 ml pipette. Five pour plates were prepared using 0.5 ml of the suspension for each plate. Tryptose-phosphate agar with peptic-digested blood was used as the medium. Colonies were counted after the plates had been incubated at 37°C for 24 hours. The experiment was repeated 10 times for a total of 50 plates.

The results of the 10 experiments have been combined into a single experience for the purpose of measuring the variation in successive samples (Figure 1). A theoretical distribution curve is also shown for comparison with the observed results. The theoretical curve shows the results expected if the only source of variation were sampling from a homogeneous suspension.[§] The agreement of observed and expected results is close, permitting the conclusion that the suspensions were essentially homogeneous.

Similar experiments were carried out with each of 3 other organisms (*Staphylococcus aureus*, *Hemophilus influenzae*, and a gram-negative coccus). Variations in plate counts were somewhat greater than expected due to sampling, but not grossly so.

The second technical difficulty investigated

[§] If the only source of variation were sampling from a homogeneous suspension the counts in a given experiment should have formed a hypergeometric distribution with a standard deviation equal to the square root of $5/6$ of the mean plate count.

—that of the need of visual aid in identifying the colonial characteristics of bacteria—is apparent from Table III. The first 2 columns show the accuracy of naked eye identification of colonies as they appeared on blood agar plates inoculated directly with throat swabs. The last 2 columns show the improvement accomplished by using the wide-field binocular microscope (magnification 15x) to examine plates prepared by the broth-suspension technic.

The advantage of microscopic examination is that it facilitates the accurate differentiation of colonial types and, therefore, leads to the detection of colonies which might have been missed by the naked eye. The fact that it makes possible an increased accuracy of naming colonies is important when a survey is being made for the presence or absence of a particular organism.

The above results indicated that the preparation of a broth suspension from a swab and the use of a microscope in reading plates provided an improved, though not yet ideal, cultural technic, and decreased considerably the errors incurred when the swab was rubbed directly on the culture medium. The data further indicate, however, that the inoculation of only a single plate from the broth suspension, in contrast to multiple plates, considerably decreases the likelihood of determining reliably the presence or absence of an organism of relatively low frequency.

Quantitative growth and nutritional interdependence. The preceding data indicated that a broth-suspension of a single bacterial species can be prepared that for practical purposes is homogeneous, as indicated by growth on the surface of a plate. Similar experiments were then carried out using a mixture of pure cultures of 2 species of bacteria to determine whether or not the growth of each would be quantitative. If such were the case, the colonies of the 2 species should be scattered at random over the surface of the medium. In one set of experiments, *S. aureus* and *beta*-hemolytic streptococci were used; in the second set, *H. influenzae* and *beta*-hemolytic streptococci were employed, because of the nutritional dependence of *H. influenzae* on

TABLE V.
Comparison of Two Methods for Detecting *Beta*-hemolytic Streptococci.

	No. positive by Pike method	No. negative by Pike method	Total
Positive by direct method	21	3	24
Negative by direct method	31	195	226
Total	52	198	250

TABLE VI.
Number of Isolations of *Beta*-hemolytic Streptococci from Men Cultured Three Times.

Group and type of streptococcus	1st culture (direct)	2nd culture (Pike)	3rd culture (direct)
Group A, no type*	47	118	48
" A, typable	19	24	7
" B	3	63	1
" C	15	49	27
" F	1	3	5
" G	7	32	12
No group	3	4	2
Not done	1	0	1
Total	96	293	103

* Sera for types 1, 2, 3, 4, 5, 6, 10-12, 11, 14, 17, 18, 19, 22, 23, 24, 26, 28, 29, 30, 31, 33, 34, 36, 37, 38, 39, 40, 41, 42, 43, 44, 46, and 47 were employed throughout most of the study. Types 8, 9, 13, 15 and 32 were available for part of the period.

streptococci, two studies were made to test the advantage of Pike's selective medium for this organism.^{20,21}

In the first study a total of 250 throat swabs was obtained by culturing 125 well soldiers on each of two occasions. Each swab was streaked directly on a blood agar plate, and then treated according to Pike's method. The results are shown in Table V.

The superiority of the selective medium is shown by the fact that *beta*-hemolytic streptococci were found by Pike's technic in 52 of the 250 swabs (20.8%), but in only 24 (9.6%) by the direct plating method. The Pike method detected the organism from 31 swabs which the direct plating method missed, while 3 swabs were positive by the direct method of plating and negative by the Pike method.

In the second study cultures were obtained on 3 occasions from a battalion of approximately 1050 men. The first and third cultures, obtained 3 weeks apart, were rubbed directly onto blood agar plates. The second cultures, taken during the intervening period,

were treated by the Pike technic. Grouping of *beta*-hemolytic streptococci was performed by the capillary tube precipitin technic, using commercial rabbit serums and bacterial extracts prepared by Fuller's method.²² Typing was done by the capillary tube modification of the Lancefield method.²³ ¶

Table VI shows the number of isolations of *beta*-hemolytic streptococci in men who were cultured 3 times. The swabs examined by Pike's method showed an increased number of streptococci in every group. The greatest increase was for Group B, the least for typable strains of Group A. Since the 3 cultures were not taken simultaneously, the results are not strictly comparable. Several facts, however, suggest that the conclusions are valid. The Pike cultures were interposed between the other two during a period when

²² Fuller, A. T., *Brit. J. Exp. Path.*, 1938, **19**, 130.

²³ Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, **78**, 127.

¶ We are grateful to Drs. Homer F. Swift and Rebecca C. Lancefield for generous supplies of typing sera, capillary tubes, and serum containers; and to Dr. Chester S. Keefer, Director, Commission on Streptococcal Infections, Army Epidemiological Board, for additional sera.

²⁰ Pike, R. M., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 186.

²¹ Pike, R. M., *Am. J. Hyg.*, 1945, **41**, 211.

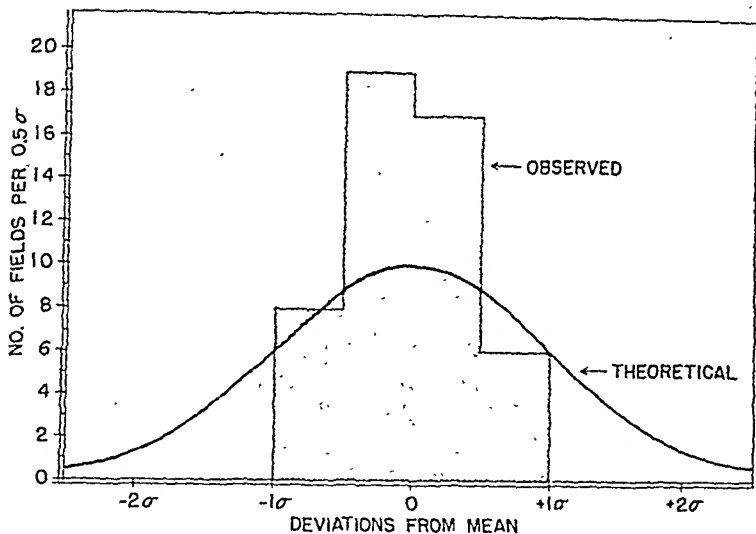


FIG. 2.
% distribution of fields of 100 colonies of a mixture of *Beta*-hemolytic streptococci and *H. influenzae*, according to number of streptococci per field.

of -1.5 standard deviations or greater was 10 while actually only one was observed. Again at the other end of the scale, fields with a relatively high proportion of streptococci were observed less frequently than was expected.

Such lack of variation between the differential counts in various fields is indicative of bias. It suggests that the colonies grew as if the organisms had been placed in relationship to one another in an orderly distribution and not at random, and indicates a relationship, nutritional or otherwise, between the numbers of bacteria of each species which develop into colonies. Probably the presence of one species enhanced the growth of the other.

The second set of experiments was performed in the same manner with a mixture of pure cultures of *H. influenzae* and *beta*-hemolytic streptococci. A total of 50 fields was counted differentially. The lack of variation in differential counts (Table IV and Figure 2) was more marked than with the mixture of *beta*-hemolytic streptococci and *S. aureus*, indicating even a greater dependence of one upon the other.

These observations suggest that the growth on a plate is a resultant of the nutritional

inter-relationship of the organisms and the adequacy of the media. The total number of colonies of a given species may be influenced to an undetermined degree by the relative presence or absence of other species. With the large variety of bacteria found in a throat culture this relationship is probably very complex, since not only symbiosis but also antagonism may be involved. Unless these relationships can be circumvented, possibly by selective media or by media adequate for the nutritional demands of all pharyngeal flora, it seems impossible to interpret quantitatively the growth which appears on the usual culture plate.

Thus the absolute or relative growth of colonies on the plate does not necessarily reflect the occurrence and distribution of bacteria in the pharynx. This consideration assumes importance in trying to determine whether organisms normally present in the throat are increased in number in the presence of infection.

Use of a selective medium for detecting beta-hemolytic streptococci. Selective media as yet provide a means of detecting the presence of only a limited number of bacteria found in the upper respiratory tract. Because of special interest in *beta*-hemolytic

The Effect of ACTH Upon the Blood Ketone Bodies of a Fasted Normal Dog

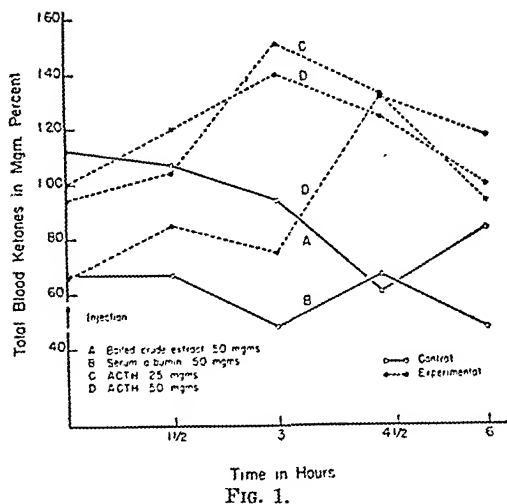


FIG. 1.

of Weichselbaum and Somogyi³ on 2 ml samples of blood. Blood was taken by veni-

puncture and in most instances the determination was done in duplicate.

Results. The data from the experiments are presented graphically in Fig. 1. It will be seen that in each case in which adrenocorticotrophic hormone was given there was an increase in ketonemia which reached its peak at either 3 or 4½ hours after injection and subsequently declined.

In neither of the control experiments was there a rise in the level of blood ketone bodies. Although the order of magnitude of the change was small it was a large percentage increase in view of the low initial levels. In view of the consistency of the response it was felt to be significant.

Conclusion. Adrenocorticotrophic hormone produced an increased ketonemia in a fasted normal dog.

³ Weichselbaum, T. E., and Somogyi, M., *J. Biol. Chem.*, 1941, **140**, 5.

16615

A Simple Method for Repeated Bone Marrow Aspirations in Rats.

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During the course of another investigation on the change in the hematopoietic tissues of the adult rat, it became necessary to develop a method for repeated bone marrow aspirations. Sawitsky¹ has perfected a technic for repeated bone marrow aspirations on the guinea pig which was a modification of the Sawitsky and Meyer² technic for bone marrow aspiration on cats. Vogel³ has recently

investigated the femoral bone marrow of adult rats, but was unable to obtain marrow without sacrifice of the animal; Cameron and Watson⁴ modified Vogel's technic so that repeated aspirations could be made. However, Cameron and Watson's technique requires a surgical incision. This paper will report a modification of the Sawitsky and Meyer technic adapted to rats.

Method. Twenty-six adult rats, (the first generation of a cross breeding of highly inbred Slonaker (Wistar) and Curtis Dunning rats), weighing approximately 200 g were used.

The animals were anesthetized with sufficient nembutal to produce surgical anesthesia of 20-30 minutes duration. The animal was

* National Cancer Institute Post Doctorate Research Fellow.

† This work was supported in part by the United States Atomic Energy Commission.

¹ Sawitsky, A., and Meyer, L. M., *Blood*, 1948, **3**, 1050.

² Sawitsky, A., and Meyer, L. M., *J. L. and Clin. Med.*, 1947, **32**, 70.

³ Vogel, M., *Am. J. Med. Science*, 1947, **213**, 456.

⁴ Cameron, D. G., and Watson, G. M., *Blood*, 1948, **3**, 292-4.

there was no indication of an unusual occurrence of streptococcal infection in the study group. Furthermore, the difference in carrier rate as determined by the two methods in this experiment is compatible with Pike's observations^{20,21} and with the results of the cultures of 125 well soldiers (Table V).

Summary. A technic for examining throat swabs which appears to have certain advantages is described. This method involves suspending the material on the swab in broth, inoculating blood agar plates with this suspension, and examining the resultant growth with the aid of a microscope. The experiments which led to the adoption of this technic are presented.

This and other similar technics for determining pharyngeal flora, however, have definite limitations, as indicated by the observation that inoculation of blood agar plates

with a mixture of only two bacterial species yielded colonies which were not distributed at random on the surface of the medium, but rather in a manner indicating interdependence of the organisms. Thus, the growth on a plate does not necessarily reflect the relative proportion of each species present in the inoculum.

The value of selective media as a means of detecting organisms present in low frequency has been demonstrated by utilizing Pike's medium for the isolation of *beta*-hemolytic streptococci. The carrier rate for *beta*-hemolytic streptococci was increased from 9.6%, as determined by usual cultural methods, to 20.8% by the use of Pike's method. The increase was greatest for Group B streptococci and least for the typable strains of Group A.

16614 P

Production of Increased Ketonemia in a Normal Dog by Adrenocorticotrophic Hormone.*

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Previous work¹ from this laboratory has shown that pure adrenocorticotrophic hormone increased the ketonemia of fasted normal rats. The present experiments have been undertaken to establish whether adrenocorticotrophic hormone would have a similar effect in a normal dog.

Methods. All the experiments were carried out on one female mongrel dog weighing approximately 15 kg. Two control experiments were done: one in which the dog was given 50 mg of serum albumin, the other in which the dog was given 50 mg of a crude

alkaline anterior pituitary extract which had been heated to 100°C for 1 hour. The adrenocorticotrophic hormone was prepared according to the previously published method,² and 3 experiments were done in which it was given at a dose of either 50 mg or 25 mg.

The dog was fasted 72 hours before each experiment and an interval of at least 2 weeks elapsed between experiments. An initial blood sample was taken and then the hormone or control foreign protein was administered by a single intraperitoneal injection. At 1½, 3, 4½ and 6 hours after the injection additional blood samples were taken. Total blood ketone bodies were determined by the method

* Aided by grants from the National Institute of Health, RG 409, and The Research Board, University of California.

¹ Bennett, L. L., Kreiss, R. E., Li, C. H., and Evans, H. M., *Am. J. Physiol.*, 1948, 152, 210.

² Li, C. H., Evans, H. M., and Simpson, M. L., *J. Biol. Chem.*, 1943, 149, 413.

16616 P

Influence of Postnatal Age on Kidney Function of Premature Infants.*

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In the course of an investigation on the development of kidney function, data have been obtained on the influence of postnatal age on kidney function of premature infants and values have been collected for current kidney function tests in premature infants. Measurements were made in 2 groups of premature infants all weighing between 2130 and 2520 g at the time of observation. The ages of infants in the first group (Group A) ranged from 3 to 13 days; those in the second (Group B) from 49 to 107 days.

Methods. The clearance procedures and chemically methods have been described previously.¹

Results. Mean values for inulin, urea, mannitol and para-aminohippurate (PAH) clearances and PAH tubular maxima in 17 well female premature infants are shown in Table I.† Protocols for each subject are included in the table. The individual values in Table I are calculated without correction as UV/P. For comparison with adult values, the means for each group are corrected for surface area. With this correction, the inulin clearances are 41% (Group A) and 58% (Group B) of normal adult values; the PAH clearances 25 and 34% and PAH tubular maxima 17 and 27%, respectively.

It may be seen from the data in Table I that although some overlapping occurs, mean

values for all measurements are higher in the older than in the younger infants. A marked increase in clearances is noted only in the oldest infant, ST, whose corrected inulin clearances are actually within the normal adult range. For the most part, however, the magnitude of the differences between the two groups is surprisingly small. Examination of values for individual infants gives emphasis to this latter fact: for example, at 12 days of age, PI "A" had higher values for all determinations than WI had at 66 days and higher PAH clearances than KO had at 95 days of age.‡

The C_{IN}/C_{PAH} ratios are higher in these infants than in adults. A high ratio in early infancy falling to adult values during the first few months of life has been reported.^{2,3} It is of interest that the C_{IN}/C_{PAH} ratios in the two groups of infants reported here are almost identical.

Discussion. Cessation of new glomerular formation in the kidneys of fetuses and infants weighing between 2100 and 2500 g has been reported to be dependent on the size the fetus has attained rather than related to the fact that birth has occurred.⁴ Our data on premature infants of the same

‡ Part of this wide scatter may be attributed to inaccuracy of body weight as an index of gestational age. However, the difference between the two groups in range of birth weights and postnatal ages is so great that variations in maturity related to multiple births, race, or other factors, would be minimized.

² Rubin, M. I., Bruck, E., and Rappaport, M., *Proc. Fifth International Congress of Pediatrics*, 1947, p. 99.

³ West, J. R., Smith, H. W., and Chasis, H., *J. Pediat.*, 1948, **32**, 10.

⁴ Potter, E. L., and Thierstein, S. T., *J. Pediat.*, 1943, **22**, 605.

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service. Mannitol and para-aminohippurate were supplied by Sharp & Dohme, Inc.

¹ Barnett, H. L., Hare, K., McNamara, H., Hare, R. S., *J. Clin. Invest.*, in press.

† The detailed data for inulin and urea clearances in Group A have been presented in Table Ib of a previous report.¹

TABLE I.
Differential Cell Count of the Iliac Marrow of
Normal Rats.

	Avg	Range
Myeloblast ¹	0.8	0.0- 2.0
Myelocyte	6.7	3.0-13.0
Metamyelocyte	5.1	2.3-10.6
"ring" cell	10.9	4.3-19.6
Non seg PMN	4.7	1.6-11.0
PMN	19.2	11.6-30.6
Lymphocyte	20.4	11.0-30.0
Normoblast	24.2	14.6-33.6
Erythroblast ²	1.3	0.0- 6.6
Megaloblast ³	0.0	0.0- 0.3
Eos myelocyte ⁴	2.0	0.0- 4.3
Eosinophilic "ring"	3.6	1.0- 9.0
Eosinophil ⁵	1.5	0.0- 4.6
Plasma cell ⁶	0.4	0.0- 1.3

¹ Cells of this type were seen in 23/26 animals.

² " " " " " " " " 23/26 "

³ " " " " " " " " 2/26 "

⁴ " " " " " " " " 25/26 "

⁵ " " " " " " " " 25/26 "

⁶ " " " " " " " " 21/26 "

grasped in the left hand with the thumb and middle fingers on the proximal part of the right and left femurs, respectively. A 3.0 cm No. 20 gauge spinal needle is held in the right hand so that the right middle finger is free to palpate the right posterior superior iliac spine. The needle is then inserted through the skin to the iliac bone and by a rotary motion is driven through the cortex of the iliac bone. The stylet is removed and suction applied with a dry 20 cc syringe and a drop of marrow aspirated. The syringe and needle are then removed together. The drop of marrow is placed on a clean glass slide and several smears are made directly from this drop. The smears are stained with Wright's stain. Suf-

ficient amount of marrow to do total nucleated cell counts is only occasionally aspirated. It probably is best not to try to do this as the volume removed would be too large and there might be a large admixture with peripheral blood.

Results. From each animal 300 cells were counted. The results are presented in Table I.

Discussion. Vogel has succinctly presented the cellular morphology. No essential difference has been noted between her description of the femoral bone marrow cells and the iliac bone marrow cells observed in this investigation. A few minor points have been observed. Occasionally a large cell in the red cell series—more like the megaloblast than a true erythroblast is seen and has been classified by us as a megaloblast. A somewhat higher percentage of lymphocytes is seen in our aspirations which corresponds more to the values obtained by Cameron and Watson than to Vogel's values. "Kugelhaufen" were seen in most of our smears but were too infrequent to count.

A repeat marrow may be done within 1-2 weeks on the same side. The other iliac bone may be aspirated at any time. Thus by alternating sides, bone marrow samples may be obtained at weekly intervals.

Conclusions. 1. A method for obtaining weekly bone marrow samples of adult rats is presented. 2. The bone marrow obtained from the iliac bone is essentially the same as has been obtained from the femur by previous investigators.

weight range show some increase in kidney function related to postnatal age. However, judging from the rate of development of kidney function in larger infants^{3,5} and in rabbits,⁶ the magnitude of the increase observed in our older infants may be less than would be expected in larger infants. In addition, the C_{IN}/C_{PAH} ratios did not change in our infants with increasing clearances. These latter findings suggest that development of kidney function in infants weighing up to 2500 g may be influenced by a gestational factor not operating in larger infants. A more complete de-

scription of these relationships requires direct comparison of these results with similar data on full term infants. Such observations are planned.

Conclusions. Mean values for inulin and PAH clearances and PAH tubular maxima corrected for surface area in premature infants ranged from 17 to 58% of normal adult values. Greater postnatal age appears to increase the rate of development of kidney function in premature infants weighing between 2100 and 2500 g although to a less extent than might be expected in full term infants.

⁵ Gordon, H. H., Harrison, H. E., and McNamara, H., *J. Clin. Invest.*, 1942, **21**, 499.

⁶ Williamson, R. C., and Hiatt, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 554.

We are indebted to Flora Hurwitz, R.N., for nursing and technical assistance.

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Strains of the Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico.

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(Introduced by R. E. Shope.)

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The present series of papers deals with studies on strains of the virus of foot-and-mouth disease from outbreaks in Mexico. The work was undertaken at the request of the Bureau of Animal Industry of the United States Department of Agriculture on behalf of a joint Mexican-American Commission to whom all the results recorded here have been reported.*

During the experiments recourse has been made to many new methods that have been developed at this institute, to which some preliminary reference should perhaps be made. The necessity of having a quantitative appreciation of a biological problem is becoming increasingly apparent especially in approaching problems of immunity. No great reliance can be placed on the results of "field" vaccination experiments, for example, in estimating the real value of an immunizing agent as it is not possible to analyse satisfactorily the many factors involved. This can be done only under controlled experimental conditions. Again before satisfactory interpretations can be made of the results of experiments carried out under controlled conditions it is necessary to have reliable "standard methods."

* The authors wish to express their thanks to the joint Mexican-American Commission (Lic. Oscar Flores (Mexico) and Dr. M. S. Shahan (U.S.A.), co-directors; Dr. B. T. Sims, Chief of the Bureau of Animal Industry, and Dr. Fernando Camargo, Director of the Laboratories of the Mexican Ministry of Agriculture and Livestock Industry, for the opportunity to examine the material. Their thanks are also due to Dr. H. W. Schoening in charge of the Pathological Division (B.A.I.), for his continued interest during the progress of the investigations.

The main program of the scientific staff of this Institute for the last eight years has

KIDNEY FUNCTION IN PREMATURE INFANTS

TABLE I.
Protocols and Data on Renal Clearances in Premature Infants.

Subject	Day of observation			Birth wt, g	Clearances* ml/min				P _{PAH} tubular maxima mg/min	C _{IN}	C _{PAH}
	Age, days	Wt, g	Ht, cm		Inulin	Mannitol	Urea	P _{PAH}			
Group A											
BA†	3	2130	46	2180	3.48	3.07	1.77	11.86	0.61	.29	
GU†	4	2276	46	2300	5.47	4.64	—	16.46	1.85	.32	
RA†	9	2380	47.5	2468	4.60	3.99	3.28	13.75	0.77	.33	
PI†	9	2216	47	2118	5.49	4.46	3.34	13.92	1.17	.42	
PI†	12	2320	47	2066	6.59	6.09	4.06	20.10	2.31	.32	
MA	12	2190	46	2182	4.06	3.98	2.98	15.82	1.44	.27	
RO	13	2164	45	2090	4.36	3.97	2.90	9.91	—	.42	
PA	13	2164	—	2100	3.29	3.12	2.25	—	0.69	—	
Mean: uncorrected					4.67	4.17	2.94	14.55	1.26	.34	
Mean: per 1.73 M ²					47.8	42.7	30.1	148.5	12.9	—	
Group B											
MC	49	2300	46	1360	6.38	5.64	3.75	22.09	2.28	.31	
LE	51	2360	47	1340	5.78	5.13	4.45	16.05	1.39	.31	
PR†	53	2470	45.3	1310	7.02	6.81	4.73	22.88	1.47	.30	
MB	54	2156	43.5	1018	7.33	6.19	4.52	18.77	1.62	.41	
KO	55	2404	46	1175	6.79	5.41	4.85	18.08	1.67	.33	
WI	66	2440	47	1240	5.09	—	2.67	12.77	1.56	.42	
MN	73	2420	44	960	6.06	—	2.96	20.89	1.20	.39	
KU	95	2510	47	740	6.77	—	4.74	19.20	2.84	.37	
ST†	107	2520	45	820	11.09	—	8.04	35.02	5.31	.34	
Mean: uncorrected					6.92	5.84	4.52	20.64	2.15	.34	
Mean: per 1.73 M ²					67.4	58.0	44.0	200.7	20.8	—	

* Each value represents the mean of 3 to 10 clearance periods.

† Negro.
‡ Twin.

weight range show some increase in kidney function related to postnatal age. However, judging from the rate of development of kidney function in larger infants^{3,5} and in rabbits,⁶ the magnitude of the increase observed in our older infants may be less than would be expected in larger infants. In addition, the C_{IN}/C_{PAH} ratios did not change in our infants with increasing clearances. These latter findings suggest that development of kidney function in infants weighing up to 2500 g may be influenced by a gestational factor not operating in larger infants. A more complete de-

scription of these relationships requires direct comparison of these results with similar data on full term infants. Such observations are planned.

Conclusions. Mean values for inulin and PAH clearances and PAH tubular maxima corrected for surface area in premature infants ranged from 17 to 58% of normal adult values. Greater postnatal age appears to increase the rate of development of kidney function in premature infants weighing between 2100 and 2500 g although to a less extent than might be expected in full term infants.

⁵ Gordon, H. H., Harrison, H. E., and McNamara, H., *J. Clin. Invest.*, 1942, **21**, 499.

⁶ Williamson, R. C., and Hiatt, E. P., *PROC. SOC. EXP. BIOL. AND MED.*, 1947, **66**, 554.

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Strains of the Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico.

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(Introduced by R. E. Shope.)

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The present series of papers deals with studies on strains of the virus of foot-and-mouth disease from outbreaks in Mexico. The work was undertaken at the request of the Bureau of Animal Industry of the United States Department of Agriculture on behalf of a joint Mexican-American Commission to whom all the results recorded here have been reported.*

* The authors wish to express their thanks to the joint Mexican-American Commission (Lic. Oscar Flores (Mexico) and Dr. M. S. Shahan (U.S.A.), co-directors; Dr. B. T. Sims, Chief of the Bureau of Animal Industry, and Dr. Fernando Camargo, Director of the Laboratories of the Mexican Ministry of Agriculture and Livestock Industry, for the opportunity to examine the material. Their thanks are also due to Dr. H. W. Schoening in charge of the Pathological Division (B.A.I.), for his continued interest during the progress of the investigations.

During the experiments recourse has been made to many new methods that have been developed at this institute, to which some preliminary reference should perhaps be made. The necessity of having a quantitative appreciation of a biological problem is becoming increasingly apparent especially in approaching problems of immunity. No great reliance can be placed on the results of "field" vaccination experiments, for example, in estimating the real value of an immunizing agent as it is not possible to analyse satisfactorily the many factors involved. This can be done only under controlled experimental conditions. Again before satisfactory interpretations can be made of the results of experiments carried out under controlled conditions it is necessary to have reliable "standard methods."

The main program of the scientific staff of this Institute for the last eight years has

been directed with this object in view. As a result it is now possible to measure the potency of virus suspensions of bovine origin such as are used for vaccine production by a method of titration involving the simultaneous inoculation of a number of serial dilutions of virus in the bovine tongue.¹ Sufficient observations are made for the calculation of the 50% end-point. This method guarantees the greatest accuracy commensurate with the use of a very limited number of animals.

In serum neutralization tests correlated with electrophoretic studies and the development of methods for concentrating antibodies it has been shown that by application of the above titration method it is possible to estimate with a greater degree of accuracy than was formerly attainable, the neutralizing and protective properties of bovine antisera and to detect low levels of antibody.² For quantitative estimates of this sort it is obvious that there must be basic information on the relative susceptibility of the test animals employed. In the case of foot-and-mouth disease where cattle are the experimental animals of choice for the study of virus of bovine origin, this susceptibility must vary within wide limits according to the source. There is on the one hand the stock of a country in which the disease is not endemic and in which variations in susceptibility may be attributable to other factors such as age, condition and breed and on the other hand the stock of a country in which the disease is endemic. In this case the "clean history" of the experimental animals is an extremely doubtful factor. This difficulty has been overcome in some countries *e.g.* Holland, by the importation of test animals from a country which is free from the disease.

It should be emphasized that in all the investigations at Pirbright including those under discussion, cattle with a "clean history" and

a relatively uniform level of susceptibility have been employed. While this is indispensable in estimates of virus potency and antibody levels it is equally important in the true appreciation of the limitations of tests for the non-infectivity of vaccines and in estimating the immunizing value or potency of vaccines.

Reference has been made by one of us (I.A.G.) at divers meetings to the results obtained at this Institute in developing standard methods. These included meetings held under the auspices of the Office International des Epizooties in Paris in May, 1947 and in Berne in September 1947,³ the 4th International Congress of Microbiology in Copenhagen in July, 1947 (see Proceedings) and a meeting organized by F.A.O. held at Pirbright in September, 1947. In addition to the methods mentioned above extensive observations carried out on several thousand cattle under controlled experimental conditions have shown that it is possible to estimate the relative potency of different vaccines in contact exposure tests by administering graded doses of vaccine. Results having statistical significance can be obtained, based on the calculation of 50% end-points provided that groups of an adequate number of vaccinated and untreated control cattle are employed. In such circumstances it has been demonstrated that different strains of virus have different immunogenic properties against a strictly homologous virus infection (*i.e.* infection with the same strain) with the implied necessity of selection of suitable strains for vaccination. At the meeting in Copenhagen and again at the 4th International Congresses of Tropical Medicine and Malaria held in Washington in May 1948 preliminary reports were made by one of us (I.A.G.) of some of the experiments, the results of which are embodied in this series of papers. One of the points connected with the immunity problem which has received particular attention has been the difference in antigenic behavior of strains of virus within a main immunological type group.

¹ Henderson, W. M., Thesis, University of Edinburgh, (in press as an Agricultural Research Council Report), 1945.

² Brooksby, J. B., Thesis, University of London, (in press as an Agricultural Research Council Report), 1946.

³ Galloway, Ian A., *Bull. Off. Internat. Epizoo.*, 1947, 27, 520.

Since these matters were reported another line of approach has been introduced for the detailed study of strains of virus, the application of the complement fixation test. Reference has already been made by one of us⁴ to the development at this Institute of the technic on a quantitative basis.

It is encouraging that already this necessity of developing standard methods on which so much insistence has been placed by the authors is now being widely recognized and their precepts are coming to be adopted more generally.

Paper II⁵ deals with the identification of 13 samples of virus material from Mexico and the accumulated evidence justifies the conclusion that only the virus of foot-and-mouth disease was present in any of the virus samples examined and all the methods employed for the determination of the immunological type involved indicated that all the strains could be placed in the Vallée A group.

There should be some basic information on the pathogenicity and invasiveness of the virus strains concerned before immunity studies are pursued and unless this is forthcoming it is impossible to interpret the results of vaccination experiments correctly. Paper III⁶ summarizes the observations on these characteristics of the Mexican strains of virus. Evidence is produced to illustrate the low invasiveness of these strains. The question has been discussed at some length there as it is of great interest not only because of its epizootiological significance but also in the interpretation of the results of vaccination experiments. Further points merit elaboration here. It is often exceedingly difficult if not impossible to secure any information of real value on the origin of a particular outbreak or epizootic of foot-and-mouth disease

and much of the evidence advanced does not proceed beyond the realms of speculation. In this respect the present epizootic in Mexico is no exception.

It is not known how strains of low invasiveness arise. We do not even know whether low invasiveness as determined by tests on a particular breed of cattle of fairly uniform susceptibility under a given set of conditions would be equally demonstrable if observations were made on another breed of different age and conditions in a different climate.

It is known that some species adaptation of the virus appears to occur, for strains of virus of porcine origin sometimes show poor infectivity for cattle. One of us (J.B.B.) has studied such a strain recently and while it spread readily amongst swine it did not do so from swine to cattle. The supposition that the Mexican strains of virus may have originated from swine would appear to be discounted since as has been reported in Paper II,⁵ strain MP (23.12.46) showed no special affinity for swine. On the other hand in countries in Africa, South America and Asia where the disease is endemic and where also susceptible wild animals of different species may roam as in Mexico (*e.g.* deer, wild pigs, goats, llamas, tapirs, porcupines etc. as well as small susceptible rodents of different kinds) it does not seem to be a remote possibility that the disease may spread among these and then return to infect cattle in a modified form due to passage through and perhaps some adaptation to species other than cattle. Where such conditions prevail *i.e.* a virus of low invasiveness is spreading and leaving in its wake animals with a varying degree of immunity; are they not conducive to the development of "variants" and also to the persistence and smouldering of the disease perhaps in a mild atypical form which at certain periods might not be readily recognizable as foot-and-mouth disease? If a virus spreading in this way reaches say "closed" dairy herds of a more susceptible breed of cattle *e.g.* cattle of European origin, this might provide the circumstances and necessary medium for an enhancement of the invasiveness of the strain and a "flare up" which first attracts

⁴ Brooksby, J. B., PROC. SOC. EXP. BIOL. AND MED., 1948, 67, 254.

⁵ Brooksby, J. B., Henderson, W. M., and Galloway, Ian A., PROC. SOC. EXP. BIOL. AND MED., 1948, 69, 64.

⁶ Henderson, W. M., Galloway, Ian A., and Brooksby, J. B., PROC. SOC. EXP. BIOL. AND MED., 1948, 69, 66.

attention to the existence of the disease.

Turning to the problem of the interpretation of experiments on vaccination it is apparent from what has been outlined elsewhere^{6,7} that strains of low invasiveness create added difficulties. When in contact exposure tests a considerable number of untreated susceptible control cattle fails to develop visible lesions but on the other hand becomes immunized as a result of infection it is exceedingly difficult to know to what extent the resistance exhibited by the non-reactors in the vaccinated groups can be attributed to the vaccine and to what extent to the low invasiveness of the strain of virus, unless the number of animals in each group is increased beyond the limits of practicability. How much more important this problem becomes in countries where it is difficult to procure cattle of uniform susceptibility! It is known that in countries in which the disease is endemic and where it is not the practice to select animals according to age, breed and condition, it is not uncommon to find quite a number of cattle which will not react even to intradermal inoculation of the tongue with massive doses of strains of virus of high invasiveness. It would therefore not be surprising if the number of non-reactors to such inoculations was not materially increased when strains of low invasiveness such as the Mexican strains are under investigation. The significance of the virus-host relationship is further illustrated by the facts which emerge from the observations on the titration of two of the Mexican strains of virus, MP and M.1 (in suspensions prepared from the vesicular tongue lesions or in the circulating blood) referred to in Paper III.⁶

It is obvious that unless there is awareness of the influence of the factors discussed above there can be no satisfactory approach to a discussion of what are sometimes glibly referred to as "international standards".

When in vaccination experiments there are indications that the invasiveness of the strain of virus under investigation is low and the

susceptibility of the cattle is nevertheless relatively and uniformly high, it is possible as has been recorded in Paper VI⁷ to use another method of estimating the potency of a vaccine. This is dependent on observations on the occurrence or non-occurrence of secondary lesions on the lips or feet or elsewhere following upon the inoculation of virus intradermally into the tongues of groups of vaccinated and control cattle. As in contact exposure tests little reliance can be placed on observations on too small groups of cattle for as will be seen from the results secondary lesions sometimes do not develop in the untreated control cattle.

Further work is being done to find out the correlation between the results in potency tests of vaccination with graded doses of vaccine followed by exposure to infection by contact and similar potency tests in which the vaccination with graded doses of vaccine is followed by exposure to infection by inoculation of virus intradermally into the tongue or by other routes.

Particular attention must be drawn to the results of experiments which are recorded in Papers IV,⁸ V⁹ and VI⁷ illustrating the antigenic behavior of the Mexican strains of virus MP and M.1 in relation to 119 the Pirbright stock strain of virus of the main Vallée A immunological type.

It is perhaps as well to deal first with the observations which were made in vaccination experiments with virus strains MP (Mexican) and 119 (Pirbright). When it was found that the first samples of virus sent from Mexico including strain MP were of the Vallée A immunological type, an experiment was set up to determine whether a vaccine prepared from strain 119 (Vallée A) would give as good protection to cattle exposed to infection with the Mexican strain MP as to cattle exposed to infection with strain 119. From observations as yet unpublished carried

⁸ Brooksby, J. B., Galloway, Ian A., and Henderson, W. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 70.

⁹ Brooksby, J. B., Galloway, Ian A., and Henderson, W. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 74.

⁷ Henderson, W. M., Galloway, Ian A., and Brooksby, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 77.

out under strictly controlled experimental conditions on 320 vaccinated cattle and 116 untreated control cattle in a series of experiments, it has been proved that strain 119 is of high immunogenicity against strictly homologous virus infection (50 per cent end-point protection dose = 8 cc). As will be seen from the results of the recorded experiments in Paper VI,⁷ a vaccine prepared from strain 119 did not, when administered in equivalent dose, give the same measure of protection against infection with strain MP as it did against infection with strain 119.

When this result was obtained an examination of the two strains was made in cross serum neutralization tests in cattle, Paper V,⁹ and it was found that bovine anti-sera had a greater neutralizing effect on strain homologous than on strain heterologous virus. In complement fixation tests also, Paper IV⁸ differences were apparent in the antigenic behaviour of strain MP and strain 119 when anti-sera from guinea pigs infected with the respective strains were employed.

Cross serum neutralization and complement fixation tests were then carried out with strains MP and M.1 and differences were exhibited also in the antigenic behaviour of these strains both in relation to strain 119 and to one another. Since strains MP and M.1 appeared to differ from one another in their behaviour in complement fixation and serum neutralization tests an investigation of their antigenic behaviour in cross immunity or vaccination experiments was made. As will be seen in Paper VI⁷ the results of these preliminary tests were not conclusive although their trend suggested some difference between strain MP and M.1. It is conceivable that in similar experiments in which groups of cattle are tested by the same method after injection with smaller graded doses of vaccine it may be possible to bring out greater differences in the antigenic behaviour of these strains. It is obvious that further work is necessary to clarify the position and that all due caution must be exercised in drawing conclusions. However, even at this stage, there is justification for the statement that the results of the examination of the antigenic behaviour of the Mexican strains of virus

has strengthened the evidence for the occurrence of "variants" within the main Vallée A immunological type group. The use of the term "variant" is one of convenience only and in this instance is for the purpose of describing some observed difference in the antigenic behaviour of strains of virus which are so similar in their behaviour in general immunological typing tests that they can clearly be classified only in one particular type group. This class of "variant" is distinct from others apparently more complex which has been described by Traub and his co-workers^{10,11} as the result of complement fixation tests. These latter variants show some partial relation to other immunological type groups. There appears to be correlation in some instances at least between the differences brought out by the three methods of study of antigenic behaviour but a considerable amount of work is now necessary to examine as many strains of virus as possible to see whether this is universally the case. Particular attention is being paid to the strains of virus regularly employed for vaccine production in the different European centers. It would be tempting to assume that because agreement in the results of complement fixation, serum neutralization and cross-immunity tests has been observed in the experiments recorded here it would be possible to forecast the results of vaccination experiments with a strain of virus from the results of the other two simpler tests. It is premature to assume that this would be so. It would be reasonable to suppose that some of these antigenic differences may be quantitative and that the limited range of antigenicity of certain strains may be compensated for by increasing the amount of antigen administered in vaccination or again some of the difficulties may be overcome by the selection of strains of good immunizing power and of a wide range of antigenicity. The real significance of the occurrence of these "variants" within an immunological type group has yet to be assessed. What is strongly suggested

¹⁰ Traub, E., and Rodriguez, R., *Zbl. Bakt. Abt. 1, Orig.*, 1944, 151, 380.

¹¹ Traub, E., and Möhlmann, H., *Berl. und Münch. tierärztl. Wschr.*, 1946, 1, 1.

is that these observations cannot be ignored in immunization procedures since Traub and Möhlmann¹¹ while working in Europe recovered some similar Vallée A "variants" from some cattle which developed the disease although they had been vaccinated in the field with a stock bivalent vaccine in which two strains of virus, one of the Vallée O type, and the other of the Vallée A type were incorporated. The cattle in these "vaccine breaks" had been vaccinated with a Schmidt-Waldmann aluminum hydroxide vaccine.

Their method of approach to the problem was different from that of the present authors in that they came across these "variants" while carrying out complement fixation tests. They did however add a rider to the effect that although the late war had prevented them from being in a position to place their observations on record, they had found that the Vallée A strain of virus used for vaccine production was not so immunogenic against the other "variants" as it was against itself and the same held true for the behaviour of the other "variants."

It is apparent, and this was stressed at the 4th International Congress of Microbiology in Copenhagen, that the position with regard to strains of virus of foot-and-mouth disease is similar to that which appears to exist with strains of influenza virus *i.e.* "variants" occur within a main immunological type group. Where workers on foot-and-mouth disease have an advantage over those engaged on the influenza problem is that although for obvious reasons progress is bound to be slower, the strains of virus can be studied in the host from which they were recovered and cross immunity vaccination experiments involving exposure to infection with a selected strain of virus can be carried out on cattle in which the disease occurs under natural conditions.

In the present series of experiments the Mexican strains MP and M.1 and the stock strain 119 were all strains of bovine origin. The serum neutralization tests were made with bovine antisera and these and the cross immunity tests were carried out in cattle. Until some means is devised of overcoming the dif-

ficulties encountered in using bovine antisera in complement fixation tests, virus strains have to be adapted to guinea-pigs for the preparation of antisera for this purpose. It would be preferable to avoid this procedure. As a result of the observations recorded above it was recommended to the Bureau of Animal Industry of the U. S. Department of Agriculture for the benefit of the joint Mexican-American Commission that until further information became available on the range of immunogenicity and specificity of other Vallée A type strains of virus it would be preferable to use Mexican strains of virus for vaccinating in Mexico (Results of experiments in which cattle injected with vaccines prepared from Mexican virus strains were subsequently exposed to infection with the same strains are recorded in Paper VI⁷). When again from the results of experiments some antigenic differences appeared to exist between the Mexican strains MP and M.1 it was recommended that until further information was obtained both these strains should be incorporated in vaccines for use in Mexico, in fact a bivalent vaccine incorporating MP and M.1 should be employed. The position is however in a constant state of flux and it must be reviewed from time to time.

This is neither the time nor the place to discuss fully the observations recorded in this series of six papers in relation to the control of foot-and-mouth disease. The procedure must be governed by the conditions obtaining. Attention might however be drawn to the more obvious implications. Every effort should be made to prevent foot-and-mouth disease becoming endemic in a country. This can be accomplished in certain exceptional circumstances by a "stamping-out" policy alone involving the early and rapid slaughter and disposal of carcasses by burial or burning and the application of a "cordon sanitaire" including police and quarantine measures.

If vaccination has to be introduced, owing to the failure or inapplicability of a "stamping out" policy, either alone or in combination with slaughter in zonal control measures or on a more extended basis then rapidity is

again the key to the situation and every effort should be made to induce as high a grade of immunity in as many susceptible animals as possible in as short a time as possible.

Whatever the class of vaccine to be employed, there must be information obtained on the strain or strains of virus responsible for the outbreaks.

Furthermore a phase has now been reached in research on the disease which points to the necessity of "standard methods" such as those referred to above so that as highly effective vaccines as possible can be produced. This involves the selection of strains of good immunogenicity and of as wide a range of group antigenicity as possible. The last word on vaccination against foot-and-mouth disease has not yet been said. If when the disease is already, or should owing to untoward circumstances, become endemic new aspects of the control problem arise but this theme cannot be developed here. However something should be said about one or two points of interest. Under epizootic or endemic conditions is it not likely that some reported cases of "reinfection" in herds which were known to have passed through the disease only a relatively short time before may be due to infection, with the same strain of virus, of cattle which escaped the disease before, or at least acquired only a low grade of immunity due to the low invasiveness of the strain? Another possibility is that a strain "variant" may have arisen. It is often assumed without further investigation that such cases of so-called "reinfection" are due probably to

another immunological type. In at least one specific case of reported "reinfection" during the Mexican epizootic such a belief was not substantiated by the examination of the virus sample collected. This aspect requires further study.

Examination of samples of virus from outbreaks during the course of an epizootic has of necessity in the past been very fragmentary. The observations recorded here suggest strongly the advisability of a more detailed and continued examination of as many virus samples as possible. This would contribute to a better understanding of the epizootiological picture and the sequence of events. Indeed it would be essential if effective control and possibly ultimate elimination of the disease was the aim in view.

Nothing has been said about the possibility of the occurrence of other vesicular diseases such as vesicular stomatitis or vesicular exanthema at the same time in areas where foot-and-mouth disease is spreading but that is another question.

The problem of the attempted control of foot-and-mouth disease by procedures involving vaccination is obviously more complex in some cases than is generally appreciated but not so complex that continued scientific endeavor may not introduce considerable improvements in many directions. It should be apparent also that in applying methods of control involving vaccination, continual guidance and indeed direction of the program must come from the laboratory.

Strains of Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico. Identification.

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These observations were made during the examination of samples of virus which were recovered from cattle during an epizootic in Mexico. It was reported that foot-and-mouth disease existed in Mexico in the last quarter of 1946 and now in June, 1948, fresh outbreaks of the disease are still occurring. Up to the present 13 separate samples of virus from Mexico have been examined at this Institute and as will be seen from the results recorded all of them could be classified in the main Vallée A immunological type group. After identification these virus samples were termed strains of the virus of foot-and-mouth disease. For the purpose of future reference a list is appended with the dates of collection and reported geographical origin:

- MP—Puebla, Dec. 23, 1946
- MPB—Puebla, Jan. 1, 1947
- MC—Camarones, Jan. 21
- MSL—San Lorenzo, Jan. 31
- M.1—Jalisco, Oct. 7
- M.2—Cuerrero, Oct. 11
- M.3—Toluca, Sept. 20
- M.4—Lerma, Sept. 30
- M.5—El Salto, Jalisco, Nov.
- M.6—El Bajo, Jalisco, Mar. 19, 1948
- M.7—Guzmán, Apr. 12
- M.8—Zapotiltic, Apr. 13
- M.9—Zapotiltic, Apr. 13

So far the antigenic behavior of only 2 of the virus strains MP (23.12.46) and M.1 (7.10.47) have been investigated in some detail but further work is proceeding on the Mexican strains of virus and the results will be reported later.

These studies on antigenicity which are reported in other papers of the present series include complement fixation, serum neutralization and vaccination experiments.

Identification of infective agent and gen-

eral immunological type classification.

1. Animal Species Susceptibility.

(a) Inoculation of cattle.

The respective inoculation of suspensions of the thirteen samples of material received intradermally into the tongues of cattle has produced in every case a generalized vesicular disease resembling, in its general characteristics, foot-and-mouth disease.

(b) Inoculation of horses and donkeys. Suspensions of some of the samples of material received were inoculated intradermally into the tongues of horses and donkeys as follows:

- M.1—2 horses and 2 donkeys
- M.2—2 horses
- M.3—2 horses
- M.4—2 donkeys
- MP—2 donkeys

and contrary to what might have happened if the virus of vesicular stomatitis had been involved, no lesions were observed in any instance.

(c) Inoculation of swine. Two swine were inoculated intramuscularly with a suspension of the MP strain of virus after passage in cattle, one showed a vesicular lesion on the snout on the third day and on the feet on the fourth day and the other failed to develop lesions. A third pig inoculated by scarification of the snout and intradermally into the tongue with a suspension of the MP strain of virus showed no lesions.

(d) Inoculation of guinea-pigs. It was possible to infect guinea-pigs with suspensions of virus samples MP, MPB, MC, MSL, M.1 and the 9 strains to M.9 by inoculation intradermally into the pads of the hind feet and a generalized vesicular disease developed after adaptation to this species. Reference is made later to typing tests in guinea-pigs.

2. Complement Fixation Tests. These

have been made with stock type foot-and-mouth disease and vesicular stomatitis guinea-pig antisera. Tests involving the use of "Indiana" and "New Jersey" vesicular stomatitis stock type antisera were made with the 10 "virus samples" MP, and M.1 to M.9 and all yielded negative results. These vesicular stomatitis type antisera had been tested against their respective agents and had been proved type specific.¹

Complement fixation tests involving the use of Vallée A, Vallée O and Waldmann C stock type foot-and-mouth disease antisera were carried out with all the 13 virus samples and positive results were obtained in every case, except one (MPB), with the Vallée A antiserum and not with the Vallée O and Waldmann C antisera.

The complement fixation tests were carried out in most cases with first passage material, collected from the tongue lesions of inoculated cattle, as antigen but in one instance at least (MP) a positive result was recorded when a suspension of the original material sent, in 50% glycerine phosphate at pH 7.6, was employed as antigen. Negative results were recorded in the tests with MPB both on the original sample and on the first passage cattle material but the matter was not pursued further as typing tests in guinea-pigs and in cattle had already indicated that MPB could be placed in the Vallée A type group of the virus of foot-and-mouth disease.

3. *Differential Filtration.* Strains MP and M.1 after adaptation to guinea-pigs were examined respectively by fractional filtration through graded "gradocol" (Elford) collodion membranes and the end-point of 25 μ enables a size value of 8 to 12 μ to be attributed to both. This is the size value previously recorded for all strains of foot-and-mouth disease virus examined. The size of the virus of vesicular stomatitis is about 8 times as great, viz. 70-100 μ .

4. *Inoculation into developing hen eggs.* It was shown that MP virus would not survive when inoculated on the chorio-allantoic membranes of developing hen eggs incubated

at temperatures of 35°C to 39°C (cf the virus of vesicular stomatitis which can be propagated in eggs under these conditions). Strain M.1 behaved similarly.

5. *General Immunological typing in guinea-pigs.* Five of the Mexican virus strains MP, MPB, MC, MSL and M.1 after adaptation to guinea-pigs were examined in cross immunity tests with standard type guinea-pig strains i (Vallée O) GB (Vallée A) and GC (Waldmann C) and as a result all 5 were classified as of the Vallée A type of the virus of foot-and-mouth disease.

6. *General immunological typing in cattle.* In all 28 cattle recovered from infection with known Vallée A type strains of the virus of foot-and-mouth disease have been inoculated with 8 of the virus strains under discussion viz. MP (8), MPB (4), MC (4), MSL (4), M.1 (2), M.2 (2), M.3 (2) and M.4 (2). With the first 5 strains the inoculations were made intramuscularly and none of the 22 cattle inoculated showed any lesions; 14 of these cattle were recovered from infection with the Pirbright Vallée A type stock cattle strain 119 and 8 had recovered from infection with an Argentine (A. Cor) Vallée A type cattle strain of the virus of foot-and-mouth disease. On the other hand 9 control susceptible cattle similarly inoculated respectively with the different virus samples MP (2), MPB (2), MC (1), MSL (2) and M.1 (2) all developed a generalized infection. With the remaining 3 virus strains M.2, M.3 and M.4 cattle recovered from infection with Vallée A type strain 119 were inoculated, 2 cattle for each strain, intradermally into the tongue with suspensions and although local reactions at the site of inoculation were recorded in 5 out of the 6 animals, no secondary lesions followed on the feet and elsewhere whereas a generalized disease involving these sites was observed on all of 6 control susceptible cattle similarly inoculated. In addition it may be mentioned that the relationship as regards the main group immunological typing (Vallée A) of the Mexican virus strains was further illustrated by the fact that of 8 cattle recovered from infection with the Mexican strain MP and subsequently inoculated either intramuscularly (M.1) or

¹ Brooksby, J. B., Proc. Soc. Exp. Biol. and Med., 1948, 67, 254.

intradermally into the tongue (M.2, M.3 and M.4) with 4 other Mexican strains of the virus, none developed any lesions.

Furthermore a limited number of tests were made by inoculating Mexican strains of virus into cattle recovered from infection with Vallée O and Waldmann C type strains of the virus of foot-and-mouth disease and in all of these a generalized foot-and-mouth disease infection resulted.

On perusal of the accumulated evidence it will be seen that none of it would suggest that any infective agent other than the virus of foot-and-mouth disease was present in any of the 13 samples of material collected from

cattle in Mexico and forwarded to Pirbright for examination. Moreover each of the 13 strains could be clearly classified in the main Vallée A immunological type group of the virus under discussion. The examination of these Mexican strains of virus involved inoculation of cattle, horses, donkeys, swine, guinea-pigs and developing hen eggs, fractional filtration experiments, complement fixation tests and cross immunity typing tests in guinea-pigs and cattle against stock type strains of the virus of foot-and-mouth disease and it was therefore much more exhaustive than that usually made of "unknown" samples of virus.

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Strains of Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico. Pathogenicity and Invasiveness.

W. M. HENDERSON, IAN A. GALLOWAY, AND J. B. BROOKSBY.

(Introduced by R. E. Shope.)

From the Research Institute (Foot-and-Mouth Disease Research Committee), Pirbright, Surrey, England.

A report on the identification of samples of virus received from Mexico has been made.¹

Before referring to the general effects of the Mexican strains of virus in cattle exposed to infection by inoculation, by contact with diseased cattle, or by the nasal instillation of virus, something should be said about the animals employed in these experiments.

Foot-and-mouth disease is not endemic in Great Britain but is introduced at irregular intervals, outbreaks occurring sporadically. Since the policy of control is by "stamping-out," that is, elimination of infected and "in-contact" animals by slaughter and burning (or under the exigencies of war, by burial) along with the rigorous application of other disinfection and "stand-still" orders, it is possible only to purchase cattle with a "clean" history as regards foot-and-mouth disease. The cattle used in these experiments

were Devon steers between 1½ and 2 years old, in good condition and weighing between 600-800 lbs, and they could be considered as relatively "standard."

1. Intradermal inoculation into the tongue.

In all 296 cattle have been inoculated with centrifuged suspensions or stock filtrates of the 13 strains of Mexican virus. 184 were inoculated with MP virus, 80 with M.1 virus, and the remaining 32 with the other 11 strains. 293 of these 296 reacted well, with local lesions at the sites of inoculation and generalized or secondary lesions at some or all of the usual sites of predilection, feet, and lips, in all cases except 2. These 2 were control animals inoculated with filtrates in an experiment described elsewhere.²

There were 3 cattle which failed to show lesions at the sites of inoculation. These had been inoculated with similar virus material

¹Brooksby, J. B., Henderson, W. M., and Galloway, Ian A., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 64.

²Henderson, W. M., Galloway, Ian A., and Brooksby, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 77.

to that which had produced the disease in other cattle. One of these however subsequently developed lesions at sites other than those inoculated, probably resulting from infection by contact with its box companion which had reacted. The other 2 developed local lesions on the tongue when re-inoculated with the Pirbright stock cattle strain 119 of the Vallée A type.

Such occasional failures to react to intradermal tongue inoculations of relatively large doses of virus have been observed with other strains of the virus of foot-and-mouth disease.

In general therefore Mexican strains of virus produce infection in the Pirbright cattle with the same degree of regularity as other strains when inoculated intradermally into the tongue.

However, there appears to be a tendency for delayed appearance of the secondary lesions. Moreover, although the presence of these secondary lesions on all 4 feet and sometimes the lips has been observed in a considerable number of cattle inoculated intradermally into the tongue with Mexican strains of virus, in many animals generalization has been incomplete and limited as regards the degree of involvement of the feet.

2. *Intramuscular inoculation.* Only 10 cattle have been inoculated by the intramuscular route, 3 with strain MP, 2 with MPB, 1 with MC, 2 with MSL, and 2 with M.1, and all of these reacted with a severe generalized foot-and-mouth disease infection.

3. *Titration of Mexican strains of virus in cattle.* Observations have been limited so far mainly to the MP and M.1 strains of virus and it has been found from the accumulated results of titrations of suspensions of epithelium from tongue lesions that the mean 50% end-point for Mexican strains of virus, $10^{-6.1}$ (414 observations) for strain MP and $10^{-6.2}$ (394 observations) for strain M.1, is not appreciably different from that recorded for other strains of the virus of foot-and-mouth disease, e.g., $10^{-6.8}$ for stock cattle strain 119 of the Vallée A type.

However, it has been noted that a number of titrations of Mexican strains, e.g., MP and M.1, have been noteworthy for the difference in reactions between cattle in pairs used

for this purpose. It seemed that the effect of the virus was impeded to a greater extent by any increased inherent resistance of the inoculated animal than was the case with other strains of the virus which have been studied. This would appear to be a more likely explanation than that the cattle used in these titrations were exhibiting greater variation in susceptibility than when other virus strains have been examined. It is interesting that although the mean 50% end-point of titrations of virus suspensions prepared from tongue lesions was not widely different from that observed with other strains of virus, yet the mean 50% end-point of titrations of defibrinated blood collected from cattle at the height of infection was somewhat lower, $10^{-2.4}$ (120 observations) for strain MP and $10^{-2.9}$ (60 observations) for strain M.1 as compared with $10^{-3.5}$ for our stock strain 119. Incidentally although an impression was received that individual comparative titrations of strains MP and M.1 showed in some cases a higher titer for the M.1 as compared with the MP strain, the accumulated results of all observations did not confirm this impression. The generally lower titer of the blood of cattle infected with Mexican strains as compared with that of animals infected with other strains of virus seems to fit into the picture of the lower invasiveness of Mexican virus strains examined. This lesser invasiveness was demonstrated in the first instance by a tendency to slower generalization after intradermal tongue inoculation but it is apparent to a greater degree in the experiments about to be described.

4. *Exposure of cattle to infection by contact.* As will be seen from Fig. 1, of 48 untreated susceptible cattle exposed in different experiments to virus infection with Mexican strains by contact with cattle showing well developed lesions, 20 failed to show any reactions. All the 48 cattle had been placed in contact with the virus donors from the time the latter were infected by intradermal inoculation of the tongue with a strong dose of virus, and had been left with them for a period of about 3 weeks. The important point is that they were with the virus donors throughout the whole of the acute stage of the infection.


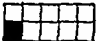

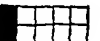
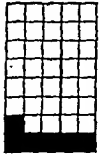

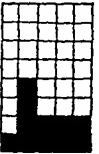
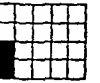

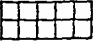

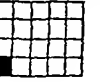
CONTACT EXPOSURE	1 DONOR PER ANIMAL. EACH PAIR IN A SEPARATE BOX	LATER TONGUE INOCULATION OF NON-REACTORS	1 DONOR PER 8 ANIMALS. ALL IN ONE BOX	LATER TONGUE INOCULATION OF NON-REACTORS
STRAIN MP VIRUS		 6 TH PASSAGE		 6 TH PASSAGE
STRAIN MP VIRUS		 16 TH PASSAGE		 13 TH PASSAGE
STRAIN MI VIRUS		 12 TH PASSAGE		 5 TH PASSAGE

FIG. 1.

Contact exposure of susceptible cattle to Mexican strain infection. Each large rectangle depicts a group of cattle with a horizontal line of five squares for each animal. The first square represents the tongue and/or lips and the next four squares the four feet. A lesion at any particular site is denoted by blackening the appropriate square. A diagonal stripe is used when lesions occurred only on the lips and not on the tongue.

When these 20 non-reactors were inoculated subsequently into the tongue with at least 10,000 ID₅₀ doses of homologous virus, 17 were shown to have developed a high grade of immunity, 11 having no reactions and 6 having reactions only at the sites of inoculation. The other 3 behaved in this severe test of resistance like unexposed fresh control cattle. It is to be noted that such development of an "inapparent infection" accompanied by a high grade of resistance to reinfection characterizes the foot-and-mouth disease pathological picture to a greater degree than is appreciated. In the writers' experience the phenomenon is much more pronounced in the case of the Mexican strains of virus under discussion than when contact exposure tests are made with other strains of virus.

In marked contrast observations may be cited on similar contact exposure tests with Pirbright cattle in which strain 119, the stock

cattle strain of the Vallée A type was investigated.

Of 72 untreated susceptible control cattle (groups of 8 in 9 different experiments) placed in contact separately in boxes with single virus donors infected with strain 119 only 2 failed to develop visible lesions. These two were proved to have contracted an "inapparent infection" by the fact that they resisted the subsequent inoculation of 10,000 ID₅₀ doses of the homologous virus. Again of 52 cattle (5 different experiments, four groups of 8 and one of 20) exposed to infection by contact with one virus donor, all developed a severe generalized infection.

It would appear to be justifiable to refer to the Mexican strains of virus as strains of reasonably good spreading power but of low invasiveness and strain 119 as one of good spreading power and high invasiveness.

Our experiments with other strains of virus have shown that some strains, e.g., 39 and 336,

may be intermediate in their invasiveness. This was evidenced in experiments in which susceptible cattle are exposed to infection for a short time or individually to single virus donors. They differ from the Mexican strains in that when groups of susceptible cattle are exposed to an accumulation of infection in one large box then they have all developed a severe generalized disease. An impression has been gained also that while certain strains of virus may exhibit lability in their spreading power and invasiveness from time to time others have greater stability in this respect. There is as yet no reliable information available on what factors, *e.g.*, method of storage, method of passage in animals, etc., are likely to affect the invasiveness of a strain. One of the difficulties lies in the setting up of an adequate organization for studies of this sort, and many strains may have been manipulated in the laboratory in one way or other to a considerable degree before being submitted to investigation. It is certain however that some strains, *e.g.*, 119 and the WA strain (a Vallée O strain which was recovered from cattle during the epizootic which spread rapidly across Europe during the period 1937-40), maintain their spreading power and invasiveness even after repeated passage in cattle by intradermal tongue inoculation or after storage in 50% glycerine for periods of from 4 to 7 years.

An attempt was made to enhance the invasiveness of the Mexican strain MP by infection of cattle by contact exposure passages but the series was broken by some of

the cattle failing to develop lesions. Another attempt was made by rapid passages in cattle by intradermal tongue inoculations (6th to 13th passage) but as will be seen from Fig. 1 the invasiveness was not increased in the tests made after the 13th and 16th passages.

Let it be mentioned also that strain M.1 recovered from cattle in a field outbreak in Mexico in October, 1947, when the disease had been spreading for about a year exhibited the same low invasiveness as strain MP recovered from cattle in Mexico in December, 1946.

5. *Intranasal instillation of virus.* It is noteworthy that this low invasiveness of strain 39 (*vide supra*) and the more pronounced low invasiveness of the Mexican strains MP and M.1 is exhibited also when single or repeated large doses of highly active virus are administered by intranasal spray and inapparent infections may develop in the same way under such conditions of experiment.

The result of one experiment with the Mexican virus strain M.1 may be cited. Four susceptible cattle received 3 doses of a gradocol membrane (Elford) virus filtrate with a titer of 10^{-4} at 24 hours interval. One animal developed lesions on the 5th day followed by a severe generalized infection involving the mouth and all four feet by the 6th day. The three others remained free of lesions and after a fortnight they were inoculated intradermally into the tongue with a filtrate of a titer of 10^{-4} . Two showed no lesions and the third had local lesions at the site of inoculation only which regressed rapidly.





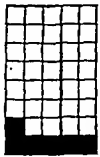

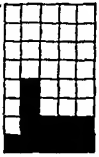



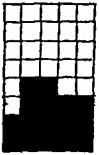

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Our experiments with other strains of virus have shown that some strains, *e.g.*, 39 and 336,

independently, also showed the value of the method. Work at this Institute has been directed at increasing the precision of measurement of the reaction, using methods already described.⁷ Application of these methods to the Mexican strains, together with the use of strictly homologous antisera, soon confirmed the existence of differences between them and strain 119, the stock Vallée A strain. The data on these observations are presented below.

Methods. The technic used follows that described in the differentiation of vesicular stomatitis and foot-and-mouth disease,⁷ and the results are presented in the same way, all end-points being referred to the dose of complement necessary for 50% hemolysis. For the differentiation of variants, it is essential to prepare a strain-homologous antiserum. So far, no success has attended experiments with bovine antisera and all results were obtained with antisera prepared against the guinea pig adapted strains.

Serum from convalescent guinea pigs was used in one or two cases but in the remainder a course of intramuscular inoculations with virus of the strains concerned was given to the convalescent guinea pigs. The antigens were 1:4 or 1:10 suspensions of infective bovine tongue epithelium, ground in M/25 phosphate buffer solution with sand. They were prepared from 4-24 hours before use and centrifuged for 20 minutes at 2,000-3,000 r.p.m. Complement and the hemolytic system were the same as in the earlier experiments.⁷ The strains used were:

TABLE I.
Complement Fixation with Vallée A Type Variants.

Antiserum	Virus strain			
	119	MP	M.1	Nil
119a	.76	.62	.62	.53
119b	.80	.61	.62	.53
MP	.51	.69	.56	.53
M.1	.52	.49	.79	.53
Nil	.50	.39	.36	.53

⁶ Rodriguez, R., Prado, M., and Palacios, R., *Bol. Soc. Paul. Med. vet.*, 1945, 7, 94.

⁷ Brooksby, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, 67, 254.

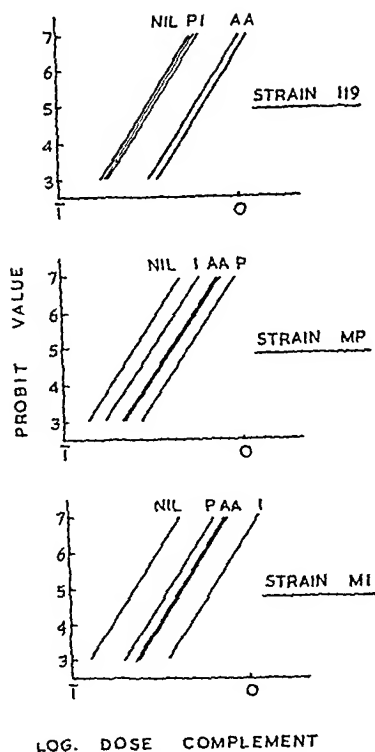


FIG. 1.
Complement-fixation with Vallée A Type Variants. The antisera used are indicated thus: A, A—Two batches of strain 119 antiserum. P—Strain MP antiserum. I—Strain M.1 antiserum.

Vallée O Type. Strain 1. A strain from cattle recovered from a field outbreak in 1924 and since maintained in guinea pigs.

Strain 39. A strain from cattle recovered from a field outbreak in 1927 and since passaged in cattle.

Vallée A Type. Strain GB. A guinea pig adapted strain received from the island of Riems, Germany, in 1929.

Strain 119. A strain from cattle recovered from a field outbreak in 1932 and since passaged in cattle.

Strain MP. A strain from cattle recovered in Mexico, December, 1946.

Strains M.1 and M.2. Strains from cattle recovered in Mexico, October, 1947.

Strain AX. A cattle-passaged strain received from the Argentine in 1944.

Waldmann C Type. Strain GC. A guinea

Strains of Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico. Complement Fixation Tests.

J. B. BROOKSBY, IAN A. GALLOWAY, AND W. M. HENDERSON.
(Introduced by R. E. Shope.)

From the Research Institute (Foot-and-Mouth Disease Research Committee), Pirbright, Surrey, England.

On the basis of cross-immunity tests, the great majority of strains of the virus of foot-and-mouth disease can be classified as belonging to one of 3 immunological types, known as Vallée O, Vallée A, and Waldmann C. It has been suspected, however, almost since the types themselves were established, that differences of a lesser degree existed in the antigenic behavior of some strains within one immunological type. Such suspicion arose from observations on cross-immunity in cattle and guinea pigs, but owing to the difficulties associated with experiments in large animals with this highly infective virus, and the deficiencies of experiments in laboratory animals, arising from possible alterations in strains during passage, precise observations are lacking. The differences between such strains within a type in any case appeared to be of small significance as compared with the very clear-cut demarcation of the types themselves.

When work was proceeding at this Institute, during 1947, on strains of virus recovered from the present epizootic in Mexico, this phenomenon of "variant" strains was encountered in a new and important connection. It was found that, although a particular strain (MP), from Mexico, could be classified as of Vallée A type, vaccination of cattle with a potent vaccine prepared from the Pirbright stock cattle strain 119 of the same type, gave poor protection against the Mexican strain. This result is reported in detail elsewhere.¹

To obtain further information on this discrepancy, opportunity was taken to apply one of the more recent methods of quantitative study of the virus and its antibody. Cross-neutralization tests were carried out as de-

scribed in a subsequent paper of this series, and the results confirmed the existence of an antigenic difference between strains MP and 119.

While this work was in progress, our attention was drawn to papers by Traub and Rodriguez² and Traub and Möhlmann.³ The authors had, in the course of examination of field strains by the complement fixation method encountered 2 classes of "variant" strains of the virus, one of them being within the Vallée A type group. Some of these strains were recovered from vaccinated cattle which had later developed the disease. They could be shown to be different in their complement fixing properties from the stock vaccine strain of Vallée A type by cross tests with guinea pig antisera prepared against the individual strains. Traub and Möhlmann drew attention to the complications introduced into vaccination against the disease by the occurrence of these variant strains and it is unfortunate that the war has prevented them from publishing in full their protocols.

The development of complement fixation as a method of "typing" the virus of foot-and-mouth disease dates from the observations of Ciuca⁴ in which guinea pig antigen and antiserum were used. Traub and Möhlmann⁵ produced reliable and consistent results with bovine virus as antigen, an advance which increased greatly the usefulness of the test. Rodriguez, Prado, and Palacios,⁶ working

² Traub, E., and Rodriguez, R., *Zbl. Bakt. Abt., Orig.*, 1944, **151**, 380.

³ Traub, E., and Möhlmann, H., *Berl. und Munch. tierärztl. Wschr.*, 1946, **1**, 1.

⁴ Ciuca, A., *J. Hyg. Camb.*, 1929, **28**, 325.

⁵ Traub, E., and Möhlmann, H., *Zbl. Bakt. Abt., Orig.*, 1943 **150**, 289, 300.

¹ Henderson, W. M., Galloway, Ian A., and Brooksby, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 77.

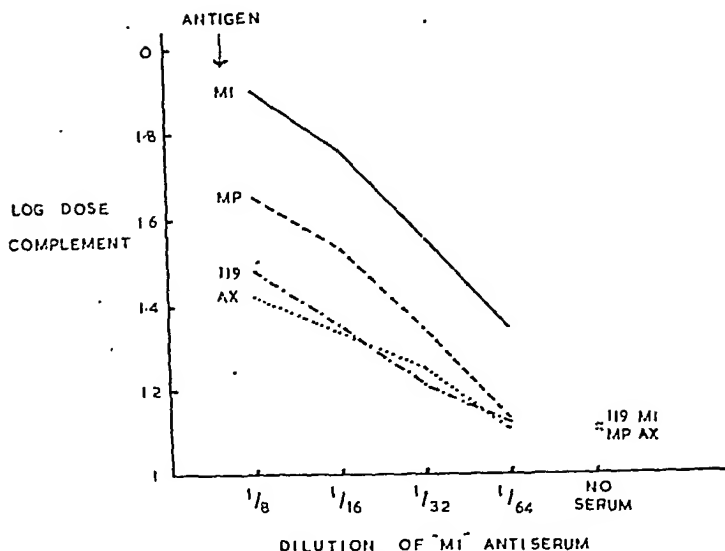


FIG. 3.

The effect of dilution of antiserum on cross-fixation with Vallée A type variants.

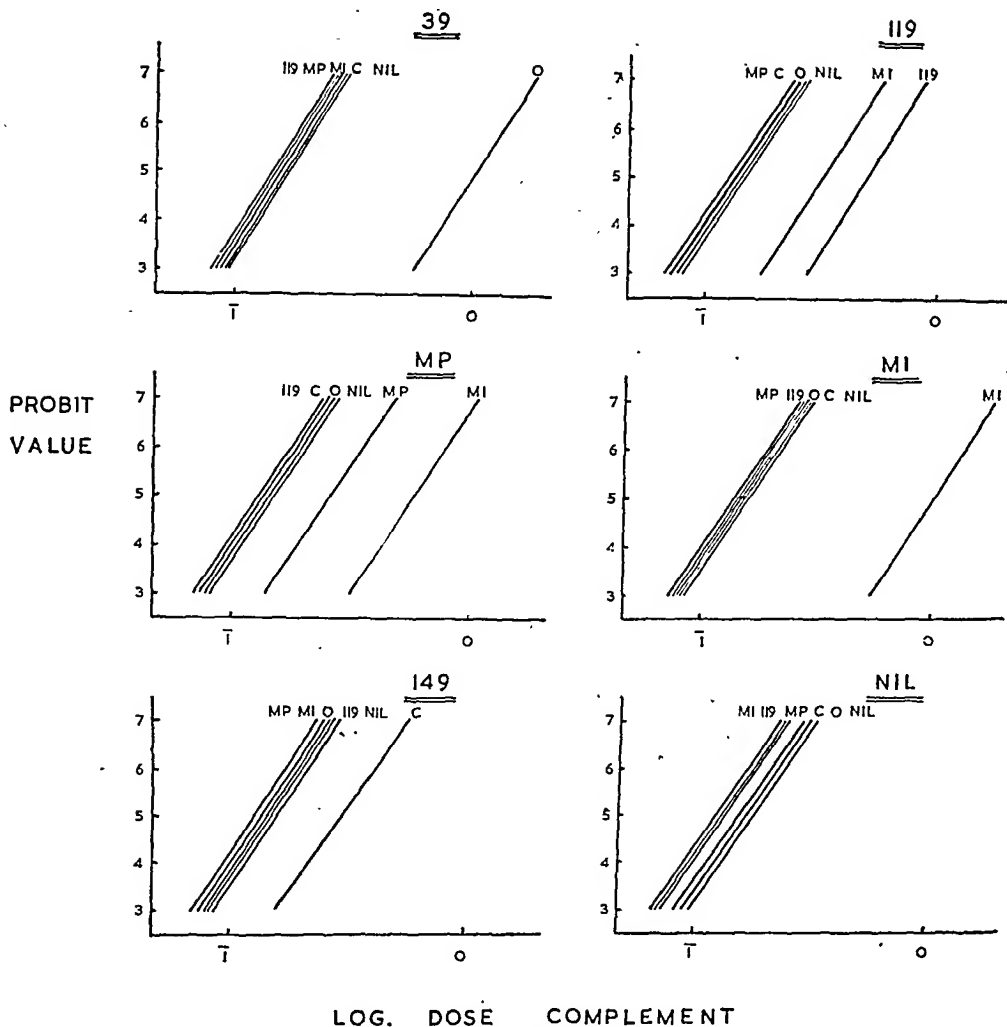
graphs in Fig. 1, it is clear that the homologous mixtures show greater fixation in each case, or, as is equally important, the relative efficacy of fixation by the various antisera depends on the antigen present. Fixation is indicated by the line concerned being further to the right.

Further observations on the relation of the variants to each other and to the immunological types are presented in Fig. 2. The result is shown of tests of strains 39, 119, MP, M.I., and 149 with the guinea pig antisera against strains 1, 119, MP, M.I., and GC. In the case of strains 39 and 149 the result is clear cut in that no serum other than the homologous shows any fixation. In the Vallée A group, strain 119 virus shows fixation, better with 119 antiserum but also with M.I. antiserum; MP virus fixes best with M.I. antiserum but also with MP antiserum, and M.I. virus shows very strong fixation with M.I. antiserum. This result would suggest that M.I. might be a "universal" A antiserum; but it is more likely to be due to a high concentration of antibody. The MP antiserum was from convalescent guinea pigs, as opposed to the other sera involved which were obtained from hyperimmunized guinea pigs. The result with MP antigen was therefore, in all

probability an expression of the low titer of the MP antiserum and high titer of the M.I. antiserum. Such anomalous effects could be counteracted by titration and suitable dilution of the serum before use in the test. This view is supported by the result shown in Fig. 3, where dilutions of the same M.I. antiserum were prepared and tested against the strain homologous virus and 3 other Vallée A antigens, 119, MP, and AX. The log. doses of complement giving 50% hemolysis are plotted against the dilutions of serum and a curve obtained by joining the points for each antigen. It is apparent from this graph that if a 1/64 dilution of serum were used in this test, fixation would be shown with only the homologous strain, the reaction with other strains of the same type having disappeared. This finding agrees well with that shown in Traub and Möhlmann's paper relating to the variants within the Vallée A immunological groups encountered by them.

In carrying out the routine type tests, the stock antisera used are from hyperimmunized guinea pigs. The aim here is to ensure fixation when testing with any strain of the same type, and for this purpose a high titer is an advantage. A test of the several variant strains of Vallée A type with the stock GB

COMPLEMENT FIXATION TESTS



LOG. DOSE COMPLEMENT

FIG. 2.

The relationship of Vallée A type variants to other strains. The antisera are indicated thus: O—Strain 1 antiserum, Vallée O type. 119—Strain 119 antiserum, Vallée A type. MP—Strain MP antiserum, Vallée A type. M.1—Strain M.1 antiserum, Vallée A type. C—Strain GC antiserum, Waldmann C type.

pig adapted strain received from the island of Riems, Germany, in 1933.

Strain 149. A strain from cattle recovered from a field outbreak in 1934 and since passed in cattle.

Results. The result of a simple experiment illustrating the detection of variants is presented in Table I and shown graphically in Fig. 1. Three antigens, from strains 119, MP and M.1, prepared as 1:10 suspensions of infective bovine epithelium, were tested against antisera from guinea pigs convalescent

from infection with these strains. Two samples of strain 119 antiserum were included. They behaved consistently. In the table, the values are the logarithmic doses of complement corresponding to the 50% hemolysis end-points in the presence of the various antigen-antiserum mixtures. The effect of the various sera on complement has been allowed for by applying a correction to all the figures in each horizontal row in the table so that the serum control series (without antigen) give the same value. From the

cattle convalescent from 14 to 21 days after the earliest lesion of the disease had appeared. Pooled samples of serum from 4 or more animals were used. The details of technique followed, in general, those described earlier.⁴ In all but one of the observations, mixtures of diluted virus and excess bovine serum were prepared, so that, for example one volume of a 10^{-2} dilution of virus filtrate was added to 9 volumes of the antiserum concerned, giving a 10^{-3} dilution in antiserum.

From 30-90 minutes after preparing the dilutions of virus in antiserum, the series were inoculated into the tongues of cattle. Four groups of 5 sites were inoculated in one animal, each group with one serial dilution. Two animals were used for each series so that 10 observations were available on each serial dilution. The calculation of the 50% end-point dilution is therefore justified. Inoculations were made when the animal was narcotized with Pentothal Sodium (Abbott),⁵ and observations on the occurrence of lesions at the sites inoculated were made at approximately 20 and 28 hours after inoculation.

The amount of virus neutralized was arrived at by deducting the titre (e.g. $10^{-2.5}$) of the virus in the presence of antiserum from the titre (e.g. $10^{-5.2}$) of the virus diluted in Hartley's Broth, giving for this example $10^{-2.7}$. The results of comparative titrations of virus in normal serum showed that little error could be attributed to non-specific effects.

An alternative method was used in one experiment described below. Dilutions of antiserum were prepared and each was mixed with an equal volume of a virus filtrate suitably diluted, as estimated on the basis of an earlier titration. In this case the dilution of antiserum corresponding to the 50% end-point was calculated from the result.

Results. The results of 4 experiments are represented diagrammatically in Fig. 1-4. On Fig. 1-3 are presented the data on the inter-

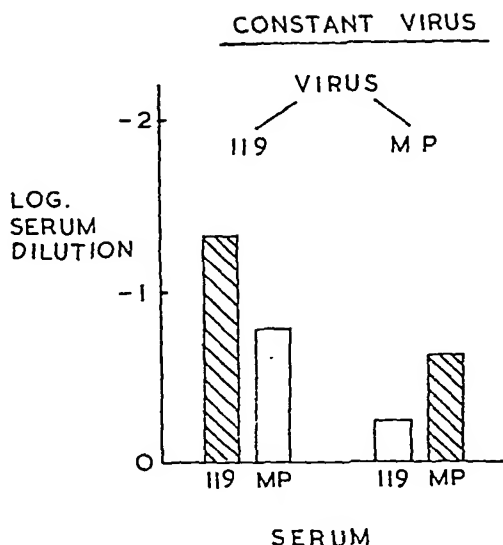


FIG. 1.
Neutralization test with viruses and antisera of two Vallée A type strains.

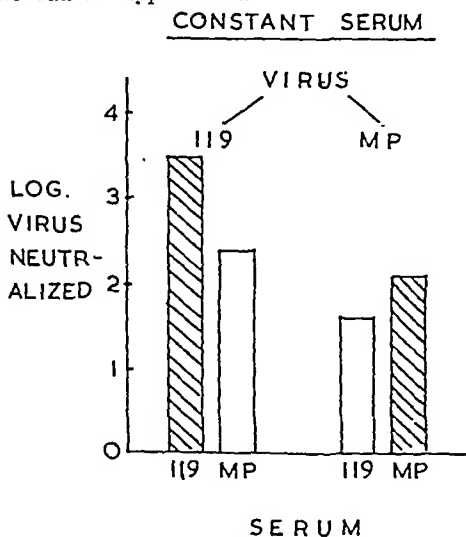


FIG. 2.
Neutralization test with viruses and antisera of two Vallée A type strains.

relationship between the Pirbright stock strain of Vallée A type, 119, and the 2 Mexican strains MP and M.1. It will be noted that for the comparison of strains 119 and MP, the observations for both serum-dilution and virus dilution methods are given. In both cases the neutralizing powers of the antisera were greatest against the corresponding strains.

⁴ Brooksby, J. B., 1946, Thesis, University of London, (in press as an Agricultural Research Council Report.)

⁵ Henderson, W. M., *J. Comp. Path.*, 1944, 54, 245.

antiserum did, in fact, give the result that all the strains fixed complement in the presence of GB antiserum, although there were at least 3 distinct variants in the group of strains used.

Discussion. The occurrence of variant strains has imposed new conditions on the successful application of the complement fixation test in foot-and-mouth disease, as well as emphasizing the importance of precision in carrying out the test. In the light of the results reported here the necessity for standardization of reagents is clear. Definition of units for antiserum and antigen must await

more extended observations. The other essential would appear to be the use of a wider range of antisera, including in most cases, one which is strain homologous. As mentioned earlier, only further work will show what differences in respect of complement fixation must be demonstrated before the differences between strains are of practical moment in immunization.

Summary. Two strains of the virus of foot-and-mouth disease, of Vallée A type, have been found to differ antigenically from each other and from the stock strain of this type used in this Institute.

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Strains of Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico. Serum Neutralization Tests.

J. B. BROOKSBY, IAN A. GALLOWAY, AND W. M. HENDERSON.
(Introduced by R. E. Shope.)

From the Research Institute (Foot-and-Mouth Disease Research Committee), Pirbright, Surrey, England.

The neutralization of the virus of foot-and-mouth disease by specific antisera has not been studied extensively. Bedson, Maitland and Burbury¹ described a technic in which guinea-pigs were used as test animals. They referred to the absence of cross neutralization between virus and antiserum of different immunological types. In examining 2 strains of the same type, they observed, in one experiment, that although the antiserum prepared against one strain neutralized to some extent virus of both strains, there was greater neutralization in the homologous mixtures. The errors implicit in their test, however, were great enough to make the validity of their conclusions somewhat doubtful. The development of a neutralization test in cattle using the method of inoculation of multiple sites on the tongues of cattle has the advantage of greater accuracy and also makes it

possible to deal with both antiserum and virus from the naturally susceptible species. Preliminary investigations suggested that this method might be used for the identification of immunological types of virus in cattle and for the detection of even finer antigenic differences. Again, as in the use of the complement-fixation test in this sphere, caution must be applied to the interpretation of results and transference of conclusions to the field of vaccination experiments. Only more extensive studies can establish the extent of the correlations to be expected between this and the other methods of measurement of antigenic differences.

Methods. The strains of virus used have been described.^{2,3} The antisera were from

¹ Bedson, S. P., Maitland, H. B., and Burbury, Y. M., *Foot-and-Mouth Disease Research Committee, 2nd Progress Report, 1927, London, p. 95.*

² Brooksby, J. B., Henderson, W. M., and Galloway, Ian A., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, 69, 64.

³ Brooksby, J. B., Galloway, Ian A., and Henderson, W. M., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, 69, 70.

the observations on strains 119 and MP. The figures for differential neutralization derived from these results are

Between strains 39 and 119.....	4.6
(Vallée O and A types).....	
Between strains 39 and 149.....	5.0
(Vallée O and Waldmann C types).....	
Between strains 119 and 149.....	3.3
(Vallée A and Waldmann C types).....	
Between strains 119 and MP.....	1.9

Thus only in the case of strains 119 and M.1 does the magnitude of a strain difference nearly equal a type difference.

Discussion. The antibody produced in response to infection appears to differ qualitatively from strain to strain of those examined. The importance of these differences in immunization may depend on a number of factors. The antibody response of an animal to an injected vaccine may not reproduce qualitatively the response to an infection even if

the preparation of vaccine from a particular strain has preserved the antigenic peculiarities of that strain. Again the quantitative factors influencing immunization may render strain specificity less important than it would at first appear. Further comment must be deferred until observations have been accumulated on these aspects. Strains shown by cross-immunity tests in vaccination experiments to be more closely related than those mentioned in this paper require to be examined, and work on these lines is continuing.

Summary. (1) Differences between strains of the virus of foot-and-mouth disease of one type have been demonstrated by cross-neutralization tests in cattle. (2) The magnitude of such differences in these experiments is not so great as that between immunological types.

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Strains of the Virus of Foot-and-Mouth Disease Recovered from Outbreaks in Mexico. Vaccination Experiments.

W. M. HENDERSON, IAN A. GALLOWAY, AND J. B. BROOKSBY.
(Introduced by R. E. Shope.)

From the Research Institute (Foot-and-Mouth Disease Research Committee), Pirbright, Surrey, England.

Experiments on the immunization of cattle against infection with strains of virus from Mexico were made to determine whether vaccines prepared from the highly immunogenic Pirbright stock cattle strain 119 of the Vallée A type would protect them against infection with these strains which are of the same type. The results of these early experiments showed a difference in the antigenic behavior of strain 119 and the Mexican strain MP. This observation led to a study of these and other Mexican strains by methods described elsewhere.^{1,2} Observations were made also on the immunizing value of vaccines pre-

pared from the Mexican strains themselves. The problem became complicated later by failure of these Mexican strains to infect susceptible cattle, in control groups included in contact infection tests, with the regularity associated with highly invasive strains.

The pattern of the experiments was the same in each case. Vaccines were prepared from different strains, groups of cattle were injected with these vaccines and 2 to 3 weeks later, the protection established was tested with the same strain or another strain of the same immunological type.

Methods. Particulars of the strains of virus

¹ Brooksby, J. B., Galloway, Ian A., and Henderson, W. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 70.

² Brooksby, J. B., Galloway, Ian A., and Henderson, W. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 74.

SERUM NEUTRALIZATION TESTS

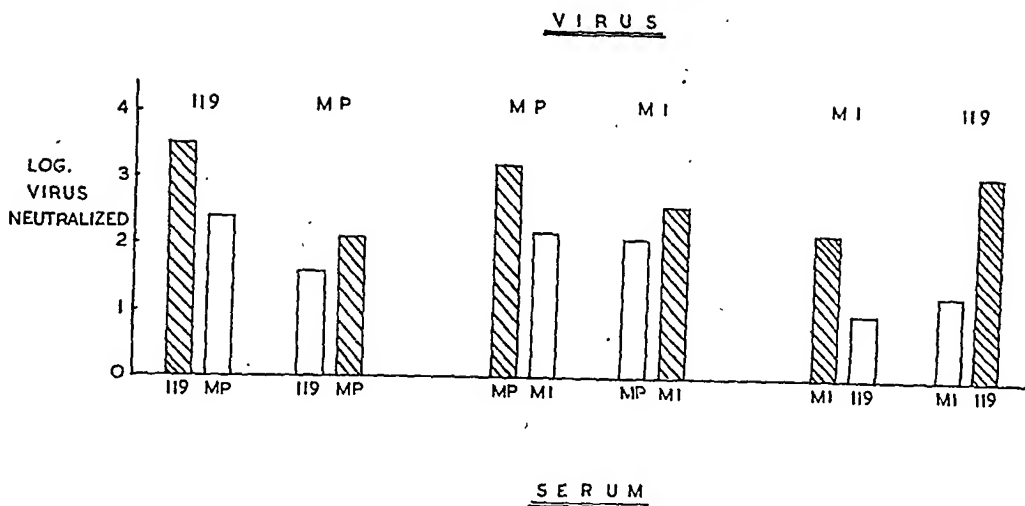


FIG. 3.

Neutralization tests with viruses and antisera of three Vallée A type strains.

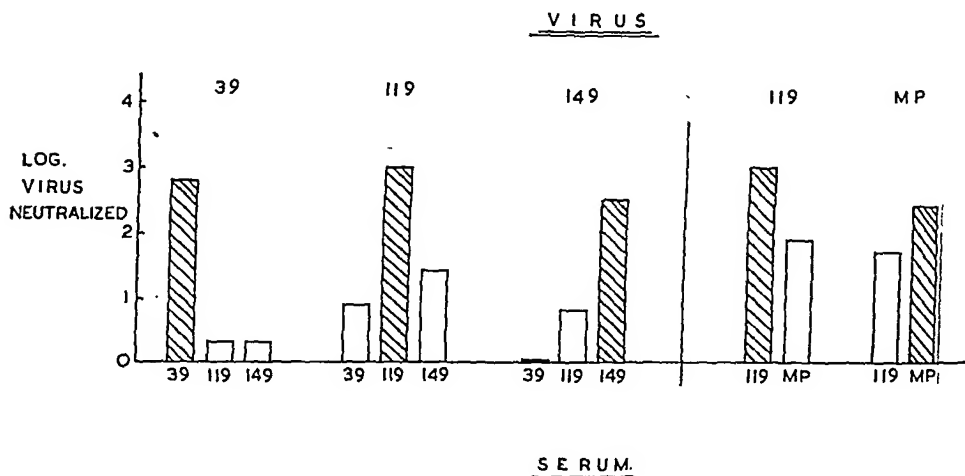


FIG. 4.

Neutralization tests with viruses and antisera of strain 39 (Vallée O type) strains 119 and MP (Vallée A type) and strain 149 (Waldmann C type).

A useful expression of the difference in relative activity of two antisera is given by the sum of the logarithmic differences in the amounts of virus neutralized by them when they are tested against first the strain corresponding to one of them and secondly the strain corresponding to the other. Thus in Fig. 2 the logarithmic difference between the amount of virus of strain 119 neutralized by strain 119 and strain MP antisera was 1.1, while for virus of strain MP, the logarithmic difference was 0.5. The sum of logarithmic

differences for these 2 antisera is therefore 1.6, and this value may be called the "differential neutralization" as between strains 119 and MP. From Fig. 3, similar calculation gives the value of the differential neutralization between strains MP and M.1 as 1.5 and between strains M.1 and 119 as 3.0.

The high figure obtained with strains M.1 and 119 led to an investigation of the differences between strains of different immunological type. The results of this experiment are represented in Fig. 4, which also repeats

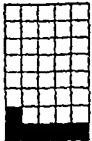



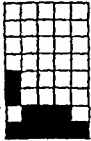
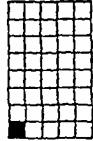

CONTACT EXPOSURE	STRAIN 119 VACCINE 10 cc.	STRAIN 119 VACCINE 30 cc.	STRAIN MP VACCINE 30 cc.	STRAIN MP VACCINE 100 cc.	CONTROLS NO VACCINE
STRAIN 119					
STRAIN MP					

FIG. 1.

Experiment A. Contact exposure test with strains 119 and MP vaccines and viruses. In this and subsequent figures each large rectangle depicts a group of cattle with a horizontal line of five squares for each animal. The first square represents the tongue and/or lips and the next four squares the four feet. A lesion at any particular site is denoted by blackening the appropriate square.

and AX. In the present experiment, 2 groups of cattle injected respectively with 10 cc and 30 cc doses of vaccine No. 93 (strain 119), were exposed to infection by contact with virus donors inoculated with strain MP virus. As a control of the potency of the vaccine another group injected with 30 cc were exposed in a similar way to infection with strain 119. Vaccine No. 94 (MP) was tested by exposing two groups of cattle injected respectively with 100 cc and 30 cc doses to infection with strain MP virus. The results of the experiment represented diagrammatically in Fig. 1 show that although vaccine 93 (strain 119) gave the expected degree of protection against the 119 strain, an equivalent amount of vaccine did not protect any of 8 cattle against infection with strain MP. A clinically recognizable disease did not develop in all of the control group of susceptible cattle exposed to infection by contact under similar conditions and this point is discussed further when the results of the next experiment are dealt with. The results show also that the protection conferred on cattle by the strain MP vaccine against infection with strain MP was not of such a high order as that given by a strain 119 vaccine against infection with strain 119.

Experiment B. Contact exposure test with strains 119 and MP vaccines and viruses.

Strain 119 vaccine No. 96—Titre of blood $10^{3.7}$.

Strain MP vaccine No. 95—Titre of blood $10^{1.5}$.

It has already been mentioned⁶ that the titre of the blood of our cattle infected with Mexican strains tended to be somewhat low. As the titration of infective material only assesses the amount of active virus, a low titre might not necessarily indicate the true antigenic level. It was for this reason that strain MP vaccine No. 95 was used in this experiment even although the titre of the blood was much lower than our usual standard for vaccine preparation.

The experiment was similar to experiment A. A dose of 100 cc of the strain 119 vaccine was injected into one group of cattle to determine whether increased dosage would compensate for the apparent antigenic dissimilarity between strains 119 and MP in exposure to infection against strain MP and another group received 30 cc doses as a link with the previous experiment.

⁶ Henderson, W. M., Galloway, Ian A., and Brooksby, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 66.

MP, M.1 and 119 used in these experiments have already been given.^{1,3}

Preparation of Vaccines. Vaccines prepared by the inactivation of infective blood were chosen for these experiments. The use of this type of vaccine as a prophylactic in foot-and-mouth disease was reported first by Graub, Zschokke and Saxer⁴ and the general principles of its preparation have since been followed at this Institute in developing inactivation tests, vaccine potency tests and for studying the difference in antigenicity of strains of virus and in other immunological problems.

Four or five litres of blood were collected from the jugular vein of each of a group of 2, 3 or 4 cattle that had been inoculated on the tongue with freshly passaged virus material.

The cattle were bled between the 24th and 48th hour after inoculation at a time when secondary lesions were developing. The blood was defibrinated, the samples pooled and a 1% solution of Du Pont "extra pure" crystal violet added to give a final concentration of 0.05%. The inactivation treatment consisted of 6 or 8 days incubation at 37°C. The titre of the pooled blood was determined by our routine method using cattle.⁵ Vaccines prepared from each new strain studied were tested for inactivation of the virus after the requisite period of incubation at 37°C. The criterion for inactivation or non-infectiveness was the recording of consistently negative results in 80 to 120 observations made on the sites of multiple tongue inoculations of a group of 8 to 12 cattle.

Vaccination of cattle. Groups of Devon steers, 1½ to 2 years old were vaccinated by subcutaneous injection into the dewlap in the region of the brisket. The vaccines were usually stored at 4°C for a short time after

completion of incubation at 37°C. In no case was this period longer than 23 days. In other experiments the potency of this type of vaccine has been proved to remain unaltered for at least 4 months at either 4°C or frozen at -20°C.

Exposure of Vaccinated and Control Cattle to Infection by Contact. The cattle were housed in loose boxes 12 feet square and in contact exposure tests one vaccinated or unvaccinated control animal was left in a box with an infected animal for at least 14 days. This virus donor was a susceptible animal which was inoculated in the tongue with virus at the time the 2 animals were placed together.

Tongue Inoculation of Vaccinated and Control Cattle. The inoculum in each case was a 0.55μ gradocol (Elford) membrane filtrate of the supernatant fluid of a centrifuged suspension of freshly collected vesicle epithelium. The filtrate was titrated in cattle and a dilution made to provide an inoculum of the desired strength. Each animal was inoculated intradermally on the tongue at 10 separate sites using 0.1 cc at each site. The result of a titration is based on similar inoculations of 0.1 cc amounts. Thus if the 50% positive end-point of the inoculum was 10⁻⁴ then each of the 10 sites on the vaccinated or control animals' tongue would receive approximately 10,000 50% end-point infective doses *i.e.* 10,000 I.D. 50.

Examination of Animals. The mouth and feet of the cattle were examined daily, at first, and later on every second or third day for 3 weeks after contact exposure and for 2 weeks after inoculation.

Results. Experiment A. Contact exposure test with strains 119 and MP vaccines and viruses. Strain 119 Vaccine No. 93—Titre of blood 10^{-3.6}.

Strain MP Vaccine No. 94—Titre of blood 10^{-3.2}.

The results of previous experiments (as yet unpublished) had shown that from 6 of 8 to 8 of 8 cattle which had received a 30 cc dose of a strain 119 vaccine resisted infection by contact with virus donors inoculated respectively with Vallée A type strains 119, A. Cor.

³ Brooksby, J. B., Henderson, W. M., and Galoway, Ian A., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 64.

⁴ Gräub, E., Zschokke, W., and Saxer, E., *Schweiz. Arch. Tierheilk.*, 1939, 81, 436.

⁵ Henderson, W. M., 1945, Thesis, University of Edinburgh, (in press as an Agricultural Research Council Report).

STRAIN 119 VIRUS	STRAIN 119 VACCINE, 100 cc.	CONTROLS NO VACCINE
TONGUE INOCULATION 10,000 ID ₅₀ AT EACH OF 10 SITES.		
CONTACT EXPOSURE		

FIG. 3.

Comparison of tongue inoculation with contact exposure of test animals in a vaccination experiment. Strain 119 vaccine and virus.

vasive strain, 119, showed that when the dose of vaccine injected is large, protection against development of secondary lesions in inoculated cattle corresponds to complete protection against contact infection.

Experiment C. Tongue inoculation and contact exposure test using strain M.1 vaccines and virus.

Strain M.1 vaccine No. 102—Titre of blood $10^{-2.8}$.

Strain M.1 vaccine No. 103—Titre of blood $10^{-2.8}$ with the addition of a filtrate of an epithelial suspension of tongue vesicular lesions to give a final titre of $10^{-3.7}$.

Two different inocula were used, one having a titre of $10^{-4.7}$ and the other a 1:1000 dilution of the first, having a titre of $10^{-1.7}$. The doses of the vaccines injected were 100 cc. The results are shown in Fig. 4. While the complete absence of secondary lesions in all the vaccinated groups demonstrates the high degree of protection that can be conferred on cattle by this type of vaccine prepared from this strain of virus, it did not permit the relative merits of the two vaccines to be assessed. Nor did it allow the demonstration of the advantage of giving 2 successive smaller doses of vaccine instead of one large one. This improved effect has been demonstrated in work (unpublished) with other strains of virus when the successive doses of vaccine were less than in the present instance.

It will be noted also that there was little difference in the effects produced respectively by the small or the large amounts of virus in the two inocula. It will be appreciated that once a primary lesion is produced the

STRAIN M1 VIRUS	STRAIN M1 VACCINES			CONTROLS NO VACCINE
	BLOOD 50+50 cc.	BLOOD 100 cc.	BLOOD+FIL- TRATE 100 cc.	
TONGUE INOCULATION 50,000 ID ₅₀ AT EACH OF 10 SITES				
TONGUE INOCULATION 50 ID ₅₀ AT EACH OF 10 SITES				
CONTACT EXPOSURE				

FIG. 4.

Experiment C. Tongue inoculation and contact exposure test with strain M.1 vaccines and virus. A white diagonal stripe denotes a secondary lesion on the lip following a primary lesion on the tongue.

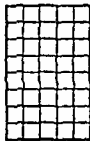


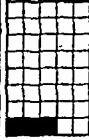
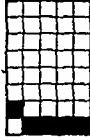
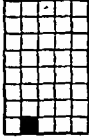
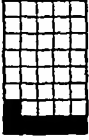
CONTACT EXPOSURE	STRAIN 119 30 cc.	VACCINE 100 cc.	STRAIN MP VACCINE 100 cc.	CONTROLS NO VACCINE
STRAIN 119				
STRAIN MP				

FIG. 2.
Experiment B. Contact exposure test with strains
119 and MP vaccines and viruses.

The strain MP vaccine was tested against infection with strain 119, as well as against infection with strain MP.

The results are shown in Fig. 2. The failure of 6 of the 8 cattle in the unvaccinated MP control group to react to infection renders negative results valueless in interpreting that part of the experiment involving exposure to infection with strain MP. The facts that 6 out of 8 controls as against the 2 out of 8 of the last experiment failed to react to exposure to infection and that 3 of the 6 non-reactors when subsequently tested for susceptibility by tongue inoculation reacted like fresh susceptible cattle suggests a change in the behavior of strain MP from experiment to experiment. This is perhaps associated with a variation in the invasiveness of the strain. The altered conditions in the present experiment no doubt account for the fact that only 1 of 8 as against 8 out of 8 cattle in *experiment A*, injected with a 30 cc dose of strain 119 vaccine reacted when exposed to infection with strain MP.

The results of the exposure of vaccinated cattle to infection with strain 119 showed that the protective value of the strain 119 vaccine was up to expectation. The complete failure of the strain MP vaccine in a dose of 100 cc to protect cattle against infection with strain 119 is unfortunately impossible to interpret as the other half of the experiment provided no satisfactory estimate of its potency against

infection with the strain homologous virus MP. More extended observations are necessary to determine whether this lack of protection of cattle by the strain MP vaccine, under discussion, against infection with strain heterologous virus 119 could be attributed to the low titre of the blood used in preparing the vaccine or was a real antigenic dissimilarity.

Two facts had arisen during the course of this work. Firstly that clinical reactors to the Mexican strains of virus did not always yield blood of adequate titre for vaccine production. Secondly that these strains were unsuitable for use in contact infection experiments owing to their inability to produce visible signs of foot-and-mouth disease in all the cattle of an unvaccinated control group.

Accordingly, in the next experiment a vaccine was prepared by adding a filtrate to infective blood to ensure having starting material of good titre. Also it was decided that the method of testing for immunity would be altered to the intradermal inoculation of the tongue with a filtrate of known virus potency in the hope that this would be followed by complete reactions in the animals of the control groups. Sufficient data have not been accumulated yet to show how the results of the tongue inoculation of vaccinated cattle can be correlated with those of infection by contact exposure tests. A preliminary experiment, Fig. 3, with a highly in-

groups were not of greater magnitude than to indicate more than a trend in the direction of some antigenic dissimilarity between strains M.1 and MP such as that strongly suggested by the results of their examination in complement fixation and serum neutralization tests. This dissimilarity may have been masked by the large dose of vaccine administered. Further observations are necessary in experiments in which a series of varying doses of vaccine will be used before full significance can be attributed to the results of the different methods of examination of strains of virus.

The results show that the lesser protection conferred by the strain MP vaccine is consistent with the lower titre of the blood used in its preparation. The "bivalent" vaccine incorporating both strains induced a comparable resistance against infection with either strain when the dose was adequate.

Summary and Conclusions. The low invasiveness of the Mexican strains of virus used in the vaccination experiments described with the resultant incomplete reaction of the control groups of cattle made the interpretation of the results difficult. Nevertheless 88 of a total of 96 cattle *i.e.* 92% which had received a dose of 100 cc of blood vaccine (with crystal violet added), in which the method of inactivation of the virus is by incubation at 37°C, showed no lesions in contact tests or no secondary lesions in tongue inoculation tests when the challenge strain or strains of virus (bivalent vaccines) were those used for preparing the vaccine. Even if all tests are included *i.e.* tests in which the doses of vaccine were 100 cc or only 30 cc or 25 cc or the challenge Mexican strain of virus was not the same as that used for vaccine production 110 of 136 *i.e.* 81% showed equivalent protection. This compared with the 12 of 56 *i.e.* 21% of non-vaccinated control cattle which remained free of all lesions in contact tests and secondary lesions in tongue inoculation tests.

As a comparison a series of observations (unpublished) with strain 119 made in a similar way may be cited. In these experiments 72 of a total of 72 cattle *i.e.* 100% injected with 100 cc of a strain 119 vaccine remained free of all lesions in contact tests and

secondary lesions in tongue inoculation tests when tested with strain 119. If 68 cattle which received only 30 cc of vaccine are added, 133 of a total of 140 *i.e.* 95% similarly remained free of lesions. These figures can be compared with 2 of a total of 88 *i.e.* 2.3% of animals in the unvaccinated control groups which similarly remained free of lesions.

These figures indicate the effectiveness of the type of vaccine employed in these experiments and give some idea of the good immunogenicity of the Mexican strains of virus.

The difference in antigenic behavior between the Mexican strains M.1 and MP was not as clear cut as those recorded in the complement fixation and serum neutralization tests.

More comparative data are required to interpret the significance of the antigenic dissimilarity between strains indicated by the different methods of examination.

It is noteworthy that although the Mexican strains under review were clearly classifiable in the Vallée A immunological group of foot-and-mouth disease virus, a potent vaccine prepared from the Pirbright stock Vallée A type strain 119 did not protect cattle against infection with strain MP virus under the conditions of the experiment.*

* Since this paper was submitted for publication the question of whether a large dose of a strain 119 vaccine would mask this antigenic dissimilarity has been investigated further. The opportunity was taken also to study the antigenic relationship of strain 119 and strain M.1. In a tongue inoculation experiment three groups of eight cattle vaccinated respectively with 100 cc doses of a strain 119 vaccine were inoculated intradermally in the tongue 2½ weeks later with strain 119, strain MP and strain M.1 viruses respectively. In the group tested with strain 119 (10,000 I.D. 50) six animals showed no reaction and two had primary lesions with no development of secondary lesions. In the group tested with strain MP (2,500 I.D. 50) and strain M.1 (2,500 I.D. 50) all of each group of eight animals developed primary lesions and, in each case, five of the eight developed secondary lesions; 1 foot, 2, 2, 4 and 4 feet respectively in the MP group and 2, 4, 4, 4 and 4 feet in the M.1 group. All the cattle in the unvaccinated control groups showed complete devel-

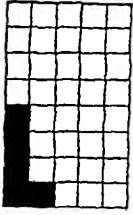




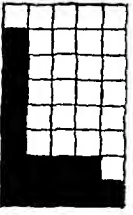
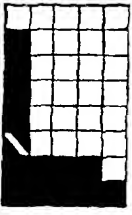

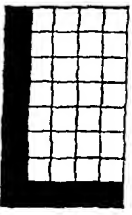

TONGUE INOCULATION	STRAIN MI VACCINE 100 c.c.	STRAIN MP VACCINE 100 c.c.	STRAINS MI+MP VACCINE		CONTROLS NO VACCINE
			50 c.c.	200 c.c.	
STRAIN MI VIRUS. 10,000 ID ₅₀ AT EACH OF 10 SITES					
STRAIN MP VIRUS. 10,000 ID ₅₀ AT EACH OF 10 SITES					

Fig. 5.

Experiment D. Tongue inoculation test with strains M.1 and MP vaccines, a "bivalent" vaccine incorporating strains M.1 and MP, and strains M.1 and MP viruses. A white diagonal stripe denotes a secondary lesion on the lip following a primary lesion on the tongue.

quantity of virus originally inoculated bears no relation to the severity of the test for the animals have then to contend with the greatly increased amount of virus released from the focus of multiplication. The non-appearance of a primary lesion in a vaccinated animal following the tongue inoculation of virus is undoubtedly of significance. Nevertheless the production of a primary lesion must constitute a severe test of the animal's immunity.

A contact exposure test was carried out at the same time and the cattle of the control groups of the tongue inoculation test were used as virus donors. Eight cattle injected with 100 cc of vaccine No. 102 were exposed to virus infection by contact and there was a group of 8 unvaccinated control cattle.

Although here again the incomplete reaction of the cattle of the control group complicates the picture to some extent, the result of the comparative test confirms that shown in Fig. 3 in the experiment in which strain 119 was used.

Experiment D. Tongue inoculation test using strain M.1 and MP vaccines, a "bi-

valent" vaccine incorporating strains M.1 and MP, and strain M.1 and MP viruses.

Strain M.1 vaccine No. 105—Titre of blood 10^{-3} .

Strain MP vaccine No. 106—Titre of blood $10^{-2.6}$.

Strain M.1 + MP vaccine No. 107—a mixture of equal quantities of vaccines Nos. 105 and 106 made at the completion of the incubation of each component.

This experiment was planned as the first of a series to determine the significance in the immunization picture of the differences recorded in the complement fixation and serum neutralization tests between strains M.1 and MP. The titres of the respective inocula of the 2 strains were approximately the same *viz.* 10^{-4} . Primary lesions were produced in the majority of the animals and the estimate of the efficacy of the vaccines was based largely on the non-development of secondary lesions. Although each vaccine gave better protection against the strain of virus used in its preparation than against the other, Fig. 5, the differences in the results between the

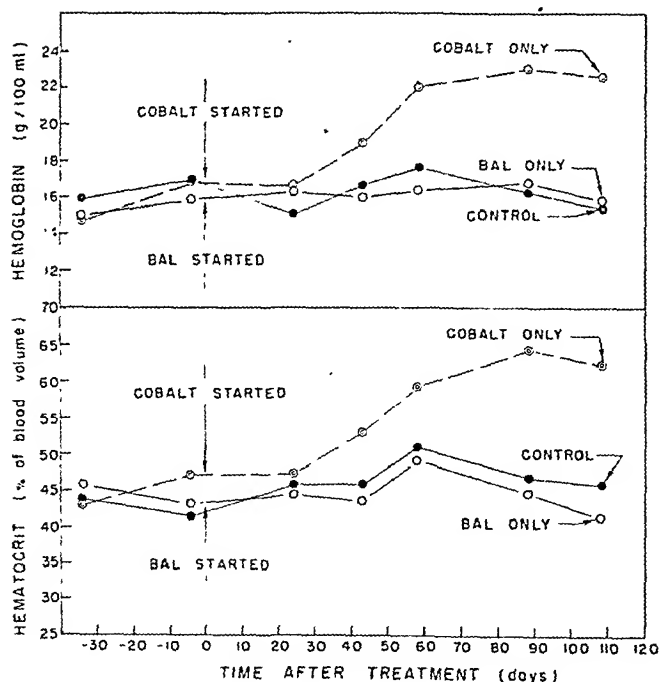


FIG. 1. Effect of 0.2 mM/kg BAL injected intramuscularly 3 times weekly and the effect of cobaltous chloride (0.357 mg calculated as Co) injected subcutaneously 5 times weekly on the hematocrit and hemoglobin values of rats.

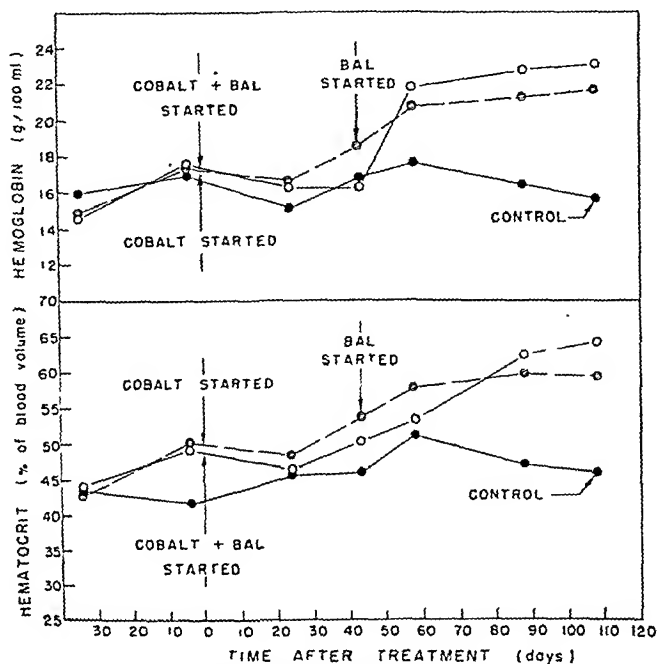


FIG. 2. Effect of 0.2 mM/kg BAL injected 3 times weekly + 0.357 mg cobaltous chloride injected 5 times weekly and the effect of cobaltous chloride + BAL, beginning 6 weeks after cobalt treatment had been initiated, on the hemoglobin and hematocrit values of rats.

This result emphasizes the necessity in attempting prophylactic control of Mexican virus infection, of testing vaccines prepared from Vallée A strains other than selected Mexican strains under controlled experimental conditions before using them in the field.

opment of primary and secondary lesions; strain

119, 16 cattle; strain MP, 8 cattle; strain M.I, 8 cattle.

This result shows that a large dose of a strain 119 vaccine is insufficient to mask the antigenic dissimilarity between strain 119 and the Mexican strains MP and M.I and that the tongue inoculation test as well as the contact exposure test can be used to demonstrate these differences in antigenic behavior.

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Effect of BAL on Cobalt-induced Polycythemia in Rats.

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Introduction. In preparation for a study of the effect of BAL (British Anti-Lewisite) on the biological effects and the excretion of radioarsenic (As^{76}), an experiment was designed to determine the influence of this chemical agent on the development of cobalt-induced polycythemia in rats. The mechanism of cobalt-induced polycythemia is not understood.

Barron *et al.*¹ demonstrated a decreased respiration (Warburg) of hemopoietic tissue from cobalt-stimulated animals. This was not corroborated by Warren *et al.*² Recently Burk *et al.*³⁻⁵ have shown that the toxic action of cobalt is related to its effect on -SH groups. It seemed worthwhile, therefore, to determine whether or not BAL, which is effective in the protection of -SH groups from arsenical poisoning, would prevent cobalt-induced polycythemia in rats.

Material and Methods. Young Sprague-Dawley rats weighing approximately 300 g each were divided into 5 groups and prepared as follows: Group I (5 rats) served as a control; Group II (5 rats) received 0.2 mM/kg of BAL in peanut oil intramuscularly 3 times weekly; Group III (15 rats) received cobaltous chloride subcutaneously 5 times weekly (0.357 mg calculated as Co); Group IV (5 rats) received 0.2 mM/kg of BAL 3 times weekly; and Group V (5 rats) received cobalt as above but the BAL was begun 6 weeks after the cobalt was initiated.

Two control hemoglobin and hematocrit (Kato) determinations were made on all rats prior to starting the BAL or cobalt injections and at biweekly or monthly intervals thereafter.

Results. As is indicated in Fig. 1 and 2 the animals which were given BAL and cobalt from the beginning of the experiment, the animals which were given cobalt from the beginning and BAL 6 weeks later, and the animals which were given only cobalt developed a comparable polycythemia (increase in hemoglobin and hematocrit values) in approximately 8 weeks. The hemoglobin and hematocrit values of control animals and animals that received BAL only remained essentially constant.

Discussion. The dose of BAL used in this

¹ Barron, A. G., and Barron, E. S. G., *Proc. Soc. Exp. Biol. and Med.*, 1937, **35**, 407.

² Warren, C. O., Schubmehl, G. S., and Wood, I. R., *Am. J. Physiol.*, 1944, **142**, 173.

³ Burk, D., Schade, A. L., Hesselbach, M. L., and Fischer, C., *Fed. Proc.*, 1946, **5**, 126.

⁴ Burk, D., Hearon, J., Caroline, L., and Schade, A. L., *J. Biol. Chem.*, 1946, **165**, 723.

⁵ Burk, D., Hesselbach, M. L., Fischer, C., Hearon, J., and Schade, A., *Cancer Research*, 1946, **6**, 497.



FIG. 1 $\times 6525$.

Large masses of sausage shaped mitochondria are scattered over entire field. Smaller black round bodies are fat droplets. Fibrous structure may be seen criss-crossing throughout picture.

whole area bounded by parallel fibrous bands. This appears to be a static representation of the streams of material which can be seen in the movies of these cells. In other areas, the great masses of mitochondria may be seen crossing those fibrous bands.

This fibrillar structure when present is clear and definite but is not present in every cell and thus may well represent a phase in either the growth or migration of the cells. Such structures have not previously been described in electron microscope studies of simi-

lar cells^{4,5†} and many types of cells will have to be studied before their general significance can be established.

Although the above findings cannot in

⁴ Porter, K. R., and Thomson, H. P., *Cancer Research*, 1947, 7, 431.

⁵ Claude, A., Porter, K. R., and Pickels, E. G., *Cancer Research*, 1947, 7, 421.

† Porter has since stated that the "cytoplasm may show a number of fine fibrils which seem to be differentiations of an otherwise homogeneous cytoplasm," *Anat. Rec.*, 1948, 100, 72.

experiment has been shown by Olcott *et al.*⁶ to be essentially nontoxic for rats when given over a period of 18 days. This dose is 10 times the minimal effective dose for the treatment of acute arsenical poisoning in cats. Since the mechanism of cobalt-induced polycythemia is not known, little can be said concerning the apparent ineffectiveness of BAL to prevent it in rats under the conditions of this experiment. The toxicity of BAL seems

to be relatively minimal since administration over a period of 3½ months produced little effect other than slightly retarding weight gain. This experiment indicates that a comparative study of the effect of radioarsenic (As⁷⁶) on cobalt-induced polycythemia in rats with or without BAL injection is feasible.

Conclusions. BAL in a dose of 0.2 mM/kg given 3 times weekly fails to prevent, cure, or materially alter cobalt-induced polycythemia in rats. The general toxicity of BAL appears minimal when given in this dosage over a 3½-month period.

⁶ Olcott, C. T., and Riker, W. F., *Science*, 1947, 105, 67.

16624 P

A Fibrillar Structure in Rat Fibroblasts as Seen by Electron Microscopy.

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During the course of comparative tissue culture studies of a strain of normal rat fibroblasts and its malignant cell derivative,¹ we obtained preparations of both of these cell types which under the electron microscope† showed an unmistakable fibrillar structure in the thinly spread cytoplasm. The fibrils, of an estimated thickness of 10 to 100 $\mu\mu$, may converge and diverge in fan-like formations of great regularity (Fig. 1) but are more often seen gathered together in long bands of varying widths (Fig. 2). The composite bands are seen clearly in phase microscope movies of the cells,³ but it is only by the in-

creased resolution available with the electron microscope that the nature of the bands becomes clear. They are apparently composed of long thin converging fibrils which in general radiate from the dense central area. That the fibrils are not produced by a wrinkling of the formvar membrane on which the cell is stretched is indicated by their uniformity, by their sharp change in direction in certain areas of the periphery, and by their fine converging and diverging structure. Besides this, no wrinkles were seen in the membrane outside of the cellular area where clear visualization is possible.

Of interest are the relations of these bands to the general structure. The mitochondria, often long and thin, seem frequently to be associated with individual fat droplets. Some of the elongated mitochondria are found in areas which show many microsomes, with the

* Supported in part by a grant-in-aid from the U. S. Public Health Service.

† Our technic of study of tissue cultured cells is essentially that of Porter² with the exception that fixation and staining has been limited to 10 minutes exposure to osmic acid vapors.

We are indebted to Warren A. Hovis for constant careful technical assistance in the preparation of the slides and photographs.

¹ Gey, G. O., *Cancer Research*, 1941, 1, 737 (abs.); Firor, W. M., and Gey, G. O., *Ann. Surg.*, 1945, 121, 700.

² Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, 81, 233.

³ Gey, G. O., Gey, M. K., Firor, W. M., and Self, W. O., *Acta Union Internationale Contre le Cancer*, 1948, in press.

formulated in terms of a continuously changing sol-gel system (Mast)¹⁰ and this concept has been further developed and applied by Lewis¹¹ to rat fibroblasts. Since gels probably acquire their rigidity from a structure of

interwoven fibers, this does not basically differ from the theory of the fibrillar structure of cytoplasm.

¹¹ Lewis, W. H., *In the Structure of Protoplasm*. A. Monograph of the Am. Soc. of Plan Phys., Iowa State College Press, 1942.

¹⁰ Mast, S. O., *Protoplasm*, 1931, 14, 321.

16625 P

The Structure of Unstained Reticulocytes.

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Brilliant cresyl blue and certain other basic dyes, if applied to fluid blood, bring out a reticulum in young red blood cells. This reticulum fails to appear if brilliant cresyl blue or other basic dyes are applied to dried and alcohol fixed blood smears. Moreover, no reticular structure could hitherto be demonstrated in the young red blood cells by microscopic examination of unstained blood with direct or oblique illumination, dark field illumination or in ultraviolet light.¹

While examining unstained wet blood films of albino mice with the phase microscope, we found that an occasional red blood cell showed a very delicate, hardly discernible reticulum. The cells showing this reticulum were fewer in number than the reticulocytes demonstrable by supravital staining with brilliant cresyl blue. However, when certain hypotonic salt solutions, preferably potassium oxalate 0.8% or ammonium oxalate 1.2% were mixed with the fresh blood, a larger number of cells, corresponding to the percentage of reticulocytes, showed distinct granules and short rods within their cytoplasm.

Most of these rods and granules showed prominent Brownian movement. In such preparation, varying number of cells showed marked loss of hemoglobin. Granules predominated in hemolyzed cells, while rods were

more prominent in cells containing ample hemoglobin. The percentage of cells showing rods or granules was higher in those areas of the preparation in which hemolysis was marked. Smears, dried and fixed in alcohol, failed to show these cytoplasmic structures. However, dried but unfixed smears, if mounted in 10% formalin or 1.2% ammonium oxalate, (Fig. 1), showed rods and granules resembling those seen in the fresh preparations. Also, fixation of dried smears in 3% aqueous potassium dichromate did not interfere with the demonstration of the rods and granules. It is noteworthy that both formalin and dichromate applied to the dried smear hemolyzed the red blood cells. Formalin added to fresh blood

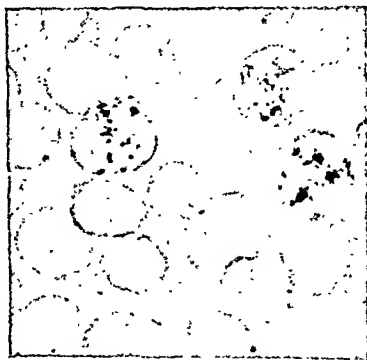


FIG. 1.

Phase illumination of dried film of mouse blood mounted in 1.2% ammonium oxalate.

¹ Isaacs, R., in Downey, *Handbook of Hematology* (New York: Paul Hoeber), 1938, 1, 16.

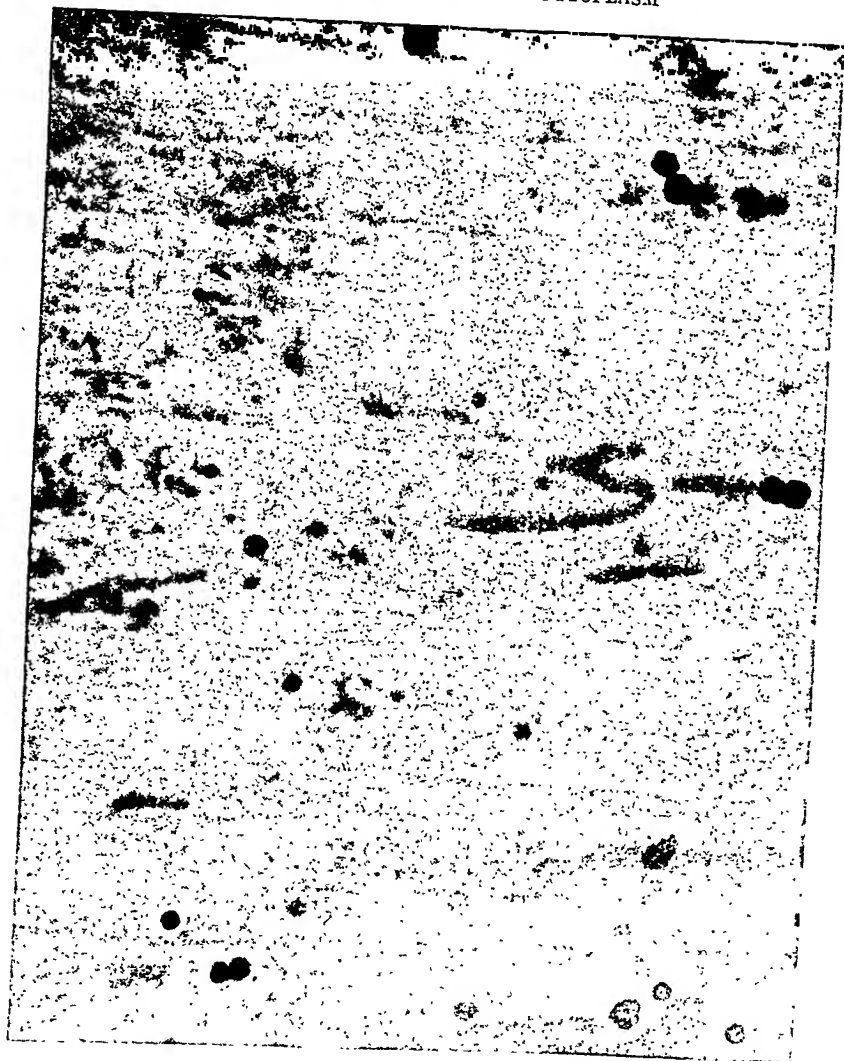


FIG. 2.

Parallel fibrous bands enclosing mitochondria and fat droplets in bed of ground substance.

themselves establish the presence of these fibrils in living cells, electron microscope studies have previously demonstrated fibrillar structure in specialized components of the cytoplasm such as the axons of nerve cells⁶ and the tails of sperm cells.⁷

Furthermore, it has been supposed for some time that cytoplasm may have a basic fibrillar structure.⁸ This is based on the demon-

stration of the elasticity of protoplasm (both to externally applied tensions and to internally displaced foreign bodies) and on the viscosity of the cytoplasm. Monné believes that the studies using the polarization microscope "prove that the ground cytoplasm of normal living cells has a fibrillar structure".⁹

The motility of *Amoeba proteus* has been

⁶ DeRobertis, E., and Schmitt, F. O., *J. Cell. and Comp. Phys.*, 1948, 38, 1.

⁷ Schmitt, F. O., In *Advances in Protein Chemistry I*, New York, Academic Press, 1944.

⁸ Frey-Wyssing, A., *Submicroscopic Morphology of Protoplasm and Its Derivatives*, New York, Elsevier Publishing Co., 1948.

⁹ Monné, L., In *Advances in Enzymology VIII*, New York, Interscience Publishers, 1948.

and, after varying periods of rest, croton oil, a non-carcinogenic agent, was used to evoke the neoplasms; in the other ultraviolet irradiation was used for both periods of treatment.

Methylcholanthrene - croton oil. Young adult strain C mice of both sexes were used. They were kept on shavings in groups of 20-24 in metal box cages with a stock grain ration and water available at all times. A solution of 0.2% methylcholanthrene in benzyl alcohol was painted on the backs of all mice twice weekly for 19 weeks. At this time approximately 10% of the animals had papillomas. The mice with papillomas were discarded and those free of visible tumors were divided into 6 groups of 42. These were then treated as follows: One group received no further treatment, another had the hydrocarbon applications continued for 13 weeks, and in a third group a 0.5% solution of croton oil in benzyl alcohol was applied to the pre-carcinogenic area, 3 times a week for thirteen weeks. The remaining 3 groups were also painted with croton oil in the same manner as the latter group except that rest periods of 4, 9, and 13 weeks elapsed between the cessation of methylcholanthrene application and the start of the treatment with croton oil. The incidence of tumors was checked every 2 weeks and they were classified as papillomas and carcinomas.

Ultraviolet Irradiation. Young adult albino strain C mice of both sexes were used. They were kept on shavings in groups of 24 in ordinary metal box cages with a stock grain ration and water available at all times except during irradiation. At the time for raying, they were transferred to a special cage 25.5 cm square by 3 cm deep, constructed of wire mesh, and divided into 24 individual compartments to prevent the mice huddling together and to minimize movements.⁹ Except for rest periods, all mice were irradiated 30 minutes a day, 6 days a week with the light of a medium pressure mercury vapor lamp. The daily amount of irradiation was 3.6×10^7 ergs/cm² at an intensity of 2×10^4 ergs/cm²

⁹ Rusch, H. P., Kline, B. E., and Baumann, C. A., *Arch. Path.*, 1941, 31, 135

TABLE I. Effect of Rest Periods on Incidence of Carcinomas Following Treatment with Methylcholanthrene and Croton Oil. (A solution of 0.2% methylcholanthrene (Mc) was applied to backs of all these mice for a period of 19 weeks prior to treatment indicated below).

Group	Subsequent	Tumor incidence—months after start of first application of methylcholanthrene						10					
		6		7		8		9		10		11	
		N	%	N	%	N	%	N	%	N	%	N	%
1	Mc continued 3 mo.	41	1	38	4	25	17	12	30	1	41	98	
2	Croton oil 3 mo.	40	2	34	7	21	18	11	28	2	35	85	
3	Rest 1 mo. then Croton oil 3 mo.	42	0	40	2	32	8	16	21	8	28	67	
4	Rest 2 mo. then Croton oil 3 mo.	42	0	40	2	35	7	21	19	12	27	65	
5	Rest 3 mo. then Croton oil 3 mo.	42	0	38	4	30	8	23	14	18	16	38*	
6	No treatment	42	0	41	1	34	5	27	9	21	14	33	

N = Free of malignant neoplasms. T = Malignant neoplasm.

* At 11 months there were: N 15, T 18, % 43

The per cent of tumors is calculated from the number of animals alive at the time the first tumor is noted.

prevented hemolysis, but also prevented the demonstration of rods and granules when blood so treated was examined in either wet or dried films.

The identity of the cells containing rods and granules with reticulocytes could be demonstrated by adding a small amount of brilliant cresyl blue or new methylene blue² to the oxalate solution used in preparing wet films. When this was done, the rods and granules were incorporated into stainable reticulum. Even small amounts of these basic dyes stained the reticulocytes very rapidly and only an occasional cell could

actually be observed in the process of staining. From such observations it appeared that some of the granules seen in unstained preparation corresponded to the metachromatic granules seen in the midst of the reticulum when stained with basic dyes.³ The reticulum, however, appeared frequently independently of the preexisting granules, although these granules may be incorporated into the meshes of the reticulum.

Preliminary experiments demonstrated essentially similar rods and granules in human reticulocytes.

² Brecher, G., unpublished data.

³ Cesaris-Demel, A., *Folia hemat.*, 1907, 4, Suppl. 1, p. 1.

16626

Influence of Interrupted Carcinogenic Treatment on Tumor Formation.*

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There is considerable evidence that the formation of cancer occurs as a series of biological changes.¹⁻⁵ This concept is supported by several types of experimental data but of special consequence is the evidence of a latent period under certain conditions of carcinogenesis. When a carcinogen is applied to the skin of mice for a time short of that necessary to induce tumors, the subsequent resumption of the carcinogenic stimulus will quickly evoke the formation of tumors even

though 2 to 3 months intervene between the 2 periods of treatment.⁶ Actually a noncarcinogenic agent, such as heat, a wound or croton oil, may substitute for the carcinogen in the second period.⁷⁻⁸ Such observations indicate that the initial application of the carcinogen produces alterations in the cells that persist for a considerable period. Whether the initial cellular changes revert gradually or persist indefinitely is not known. In order to test this point, rest periods from one to 3 months were interspersed between 2 periods during which the carcinogens were applied to mice at minimal carcinogenic levels. It was thus possible to obtain some information on the reversibility of the process.

Procedure. This investigation may be divided into two parts: in one, methylcholanthrene was employed to initiate the process

* This investigation was supported by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council and by a grant from the Jonathan Bowman Fund for Cancer Research.

¹ Berenblum, I., and Shubik, P., *Brit. J. Cancer*, 1947, 1, 383.

² Friedewald, W. F., and Rous, P., *J. Exp. Med.*, 1944, 80, 101.

³ Rusch, H. P., *Physiol. Rev.*, 1944, 24, 177.

⁴ Kline, B. E., and Rusch, H. P., *Cancer Res.*, 1944, 4, 762.

⁵ Rusch, H. P., and Kline, B. E., *Arch. Path.*, 1946, 42, 445.

⁶ Lavik, P. S., Moore, P. R., Rusch, H. P., and Baumann, C. A., *Cancer Res.*, 1942, 2, 189.

⁷ Des Ligneris, M. J. A., *Am. J. Cancer*, 1940, 40, 1.

⁸ Berenblum, I., *Arch. Path.*, 1944, 38, 233.

neoplasms was slower and the eventual number of tumors obtained was less than in the other groups; the incidence at the end of 11 months being 43%.

Ultraviolet Irradiation. The rate at which tumors appeared in the various groups of mice irradiated with ultraviolet light is given in detail in Table II. The animals remained in good health throughout the experiment and the tumors formed were identical to those previously reported.¹⁰ Mice rayed continuously for 5 months developed tumors rapidly and there was an incidence of 88% after 8 months (Group A). In the next 3 groups the 5 months of irradiation was divided into two periods with an initial irradiation of 3 months separated from the last 2 months by rest periods of one, 2 or 3 months. A rest of one month had little effect on tumor formation, and the results were essentially the same as for the control group (Group B). However, when the rest period was prolonged to 2 or 3 months, the incidence of neoplasms at 9 months was only 55 and 50% respectively (Groups C and D), but the rate of tumor formation in these groups was similar to that in Groups A and B. The rapid response to the second period of raying is evident from the data.

The effect of various other periods of irradiation or rest were also observed. When the total 5 months' irradiation was divided so that the initial 3 months of raying was followed by one of rest, one of ray, another of rest and a final one of irradiation (Group E), the results were similar to those obtained when the initial period was followed by one of rest and 2 of irradiation (Group B). Essentially no difference was seen when the final period of irradiation was only of one month duration instead of 2 (Group F). When 3 months of irradiation were divided so that the initial period of one month was separated from the final period by a month of rest, (Group G), the results were similar to those obtained when they received the same total dose over a continuous period (Group K).

Groups H, I and J illustrate that a minimal

amount of irradiation was required to induce tumors. Since this fact had been previously known these groups were employed only for controls of the present experiment. One month of irradiation by itself was insufficient to elicit tumors during the period of observation (Group I). The practical minimal time under the condition of this experiment was 2 months of continuous irradiation (Group J) and the same total dosage divided into 2 equal periods but separated by a month of rest was ineffective (Group H). Mice rayed for 3 months had a tumor incidence of 21% at the end of 9 months (Group K), and 4 months of continuous irradiation (Group L) was almost as effective as 5 months. In retrospect, it appears that the lower incidence of tumors obtained with only 2 months of irradiation would have been a more satisfactory initial dose for testing the effect of rest periods than the longer period used in this experiment. The longer initial period was selected on the basis of previous work that indicated a two month period to be a marginal one.

Discussion. The present investigation strengthens our previous suggestions that the process of tumor formation can be divided into 3 periods.^{4,5} The initial phase is a conversion of a few normal cells into a few neoplastic cells and has been called the Period of Induction^{4,5} or the Initiating Process.^{1,2} The next stage is the multiplication of such cells into growing visible tumors, and it is the division of this period into two separate stages that we wish to emphasize. The first of these periods is the Critical Period.⁵ This starts when the genesis of the neoplastic cell has been completed and is that stage in which cellular proliferation is delicately balanced. During this phase there are so few neoplastic cells present that they are more or less lost among the normal cells, and since their mass has not yet attained a size sufficient to receive a direct supply of blood, they must compete with the healthy cells for the nutrients in the tissue spaces. At such times these cells are the most susceptible to the influence of their environment; they may proliferate, lie dormant, or succumb, or all

¹⁰ Grady, H. G., Blum, H. F., and Kirby-Smith, J. S., *J. Nat. Cancer Inst.*, 1943, **3**, 371.

TABLE II.
Effect of Rest Periods on Tumor Incidence Following Treatment with Ultraviolet Irradiation.
Tumor incidence—months after start of raying

Group	Procedure (Time in months)	4			5			6			7			8			9			10		
		N	T	%	N	T	%	N	T	%	N	T	%	N	T	%	N	T	%	N	T	%
A	Ray 5	22	2	8	18	5	21	10	12	50	4	18	75	0	21	98	—	—	—	—	—	—
B	" 3, Rest 1, Ray 2	23	0	0	22	1	4	13	9	39	3	18	78	2	19	83	—	—	—	—	—	—
C	" 3, " 2, " 2	21	1	5	17	2	9	14	5	23	6	10	45	4	11	50	3	12	55	—	—	—
D	" 3, " 3, " 1	24	0	0	23	1	4	20	1	4	17	3	13	12	7	29	6	12	50	5	13	54
E	" 3, " 1, " 1	24	0	0	19	4	17	17	6	26	11	12	52	2	19	83	—	—	—	—	—	—
F	" 3, " 1, " 1	23	1	4	19	4	17	13	10	42	8	15	63	4	19	79	—	—	—	—	—	—
G	" 1, " 1, " 2	23	1	4	23	1	4	19	1	4	19	1	4	18	2	8	11	5	21	—	—	—
H	" 1, " 1, " 1	24	0	0	23	0	0	23	0	0	23	0	0	22	0	0	19	0	0	—	—	—
I	" 1, " 1, " 1	24	0	0	24	0	0	23	0	0	22	0	0	22	0	0	20	0	0	—	—	—
J	" 2, " 2, " 2	23	0	0	21	1	5	20	1	5	20	1	5	18	2	9	15	2	9	—	—	—
K	" 3, " 3, " 3	24	0	0	23	1	4	20	2	8	20	2	8	18	3	13	12	5	21	—	—	—
L	" 4	24	0	0	17	6	26	13	10	44	8	13	57	5	15	65	—	—	—	—	—	—

Designations are the same as in Table I.

/sec. The mice were examined for tumors every 2 weeks and were weighed at monthly intervals. Details of procedure for each group are given in Table II.

Results. *Methylcholanthrene-Croton Oil.* The rates of tumor formation in the various groups of mice are listed in Table I. At the end of 19 weeks of treatment with methylcholanthrene about 10% of the mice had developed papillomas. At this time, the applications of hydrocarbon were stopped and all the animals with visible tumors were discarded. The remaining mice were divided into 6 groups of 42 each and were treated according to the method indicated in Table I.

The formation of papillomas continued at a rapid rate in all groups, and one month after the initial methylcholanthrene had been discontinued, less than half of the mice in all groups were free of benign tumors. The rate of formation of the malignant neoplasms was slower and varied considerably among the groups. In the mice that received no further treatment, the rate was steadily upward and 33% of the animals had carcinomas at the end of 10 months (Group 6). The rate of development of malignant tumors was very rapid in the mice of the control group in which the hydrocarbon was continued for another 3 months (Group 1) and in the mice that were painted with croton oil immediately after the initial period (Group 2). The final incidence of tumors after 10 months in these latter 2 groups was 98 and 85% respectively. In confirmation of the earlier observation of Berenblum⁸ croton oil was almost as effective as methylcholanthrene in eliciting tumors in a precarcinogenic area. When periods of one or 2 months were allowed to elapse before the application of croton oil, the development of carcinomas was delayed slightly (Groups 3 and 4), but the rate of tumor formation paralleled that in Groups 1 and 2 following the treatment with the irritant. The final tumor incidence in groups 3 and 4 at 10 months was 67 and 65% but the slope of the curve indicated an eventual incidence close to that in Groups 1 and 2. When three months elapsed between the initial treatment and the start of croton oil, the rate of formation of

verted toward normal. It is suggested that the genesis of the neoplastic cell was completed during the initial treatment, and such cells remained dormant or divided at a de-

creased rate until further stimulated. The non-specific irritant, croton oil, exerts its effect during the critical period of carcinogenesis.

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Antigenic Relationship of *Salmonellae* to Inaba Strains of *Vibrio comma* Isolated in Egypt.

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The sera of patients infected with *Salmonellae* frequently agglutinate cholera vibrios.¹ Gohar and Makkawi,² studying *Vibrio comma* strains from the recent outbreak in Egypt, found that these organisms were agglutinated by *Salmonella enteritidis* sera. It seemed of interest, therefore, to investigate which antigenic factors are common to *Salmonellae* and cholera vibrios.

Three strains of *V. comma* of the Inaba type, isolated from the recent cholera epidemic in Egypt, were used in these experiments.* *Salmonella* "O", "Vi" and "H" sera prepared

for routine *Salmonella* grouping (typing) by methods described by Edwards a.o.³⁻⁵ were employed. Procedures recommended by Ranta and Dolman⁶ were used for the production of *V. comma* "OH" sera. Agglutination and absorption tests were carried out according to the standard methods employed for *Salmonella* grouping (typing).^{4,5}

Live, alcohol-treated ("O") and formalized ("H") antigens prepared from the cholera vibrios were tested against 25 "O", 2 "Vi" and 39 "H" *Salmonella* sera. Agglutination of the vibrios occurred with dilutions of these

TABLE I.
Results of Agglutination Tests Using *Salmonella bredeney* "O" Serum.

Agglutination (after absorption) with "O" antigen from	Serum absorbed with "O" antigen from							
		<i>S. bredeney</i> I, IV, XXVII, XII	<i>S. paratyphi</i> A I, II, XII	<i>S. pullorum</i> IX, XII	<i>S. worthington</i> I, III, XXIII	<i>V. comma</i> "k" "4" "8"		
	None							
<i>S. bredeney</i> I, IV, XXVII, XII	16*	0	6	4	4	12	12	12
<i>S. paratyphi</i> A I, II, XII	8	0	0	4	2	4	2	2
<i>S. pullorum</i> IX, XII	6	0	0	0	6	1	2	2
<i>S. worthington</i> I, III, XXIII	5	0	1	5	0	3	2	2
<i>V. comma</i> "k"	2	0	0	1	1	0	0	0
<i>V. comma</i> "4"	4	0	0	1	1	0	0	0
<i>V. comma</i> "8"	2	0	0	2	1	0	0	0

* All agglutination titers to be multiplied by 100.

¹ Felsenfeld, O., and Young, V. M., A.A.A.S. Annual Meeting, 1946.

² Gohar, M. A., and Makkari, M., *J. Trop. Med. and Hyg.*, 1948, 51, 95.

* These strains were received through the courtesy of Dr. Harry Senece of Columbia University.

³ Edwards, P. R., and Bruner, D. W., *Kentucky*

Agri. Exp. Sta. Bull., 1942, 54.

⁴ Felsenfeld, O., *Am. J. Clin. Path.*, 1945, 15, 584.

⁵ Edwards, P. R., and Bruner, D. W., *J. Bact.*, 1946, 52, 493.

⁶ Ranta, L. F., and Dolman, C. E., *Canad. J. Publ. Health*, 1943, 34, 26.

three processes may occur in the same general area at one time. The fate of small masses of neoplastic tissue depends on the balance between their proliferative capacity and the amount of stimulation or of resistance present in the neighboring tissues. The sum of all these factors determines the rate at which the cells proliferate.^{11,12} If the proliferation of the neoplastic cells continues until they attain a size sufficient to establish an independent blood supply, they then become less dependent on local environmental conditions and enter the third phase—that of Progression.⁵

The postulation of a critical period appears essential for the explanation of the results presented in this paper. In the first place, a period of reversibility is indicated because of the decreased incidence of tumors following a long interval between the first and final carcinogenic treatments. Such results may be explained on the basis of the number of cells surviving during the quiescent period. If the rest period is prolonged, some of the neoplastic cells die so that the number able to respond to the second treatment with irritant or carcinogen diminishes as the period of rest increases. Actually, if the rest period is very long, the latent tumor cells may disappear entirely or be reduced to such a level that no visible tumors could develop in the normal life of the mouse even though the proper stimulus is eventually applied. Furthermore, the evidence indicates that non-specific irritants are effective only during an interval that corresponds to the critical period described in the preceding paragraph. The irritant cannot convert normal cells into malignant ones¹ and since it also is without effect on the growth of visible tumors, it can be assumed that the action is exerted during an intermediate period. The conversion of normal cells into malignant ones is brought about solely by the specific carcinogenic agents,¹ an inference that is borne out by the

rapidity by which tumors are evoked following the treatment by croton oil. Evidence from other sources also favors this concept.^{2,5,11,12}

Indications for a critical period in this investigation are in contrast to those of Berenblum and Shubik¹ who find that the initial changes are irreversible. They used only one application of 9:10 dimethyl-1:2-benzanthracene and reported only the appearance of warts, whereas we used multiple treatments with methylcholanthrene or ultraviolet irradiation and include only the malignant neoplasms in the data of this paper. Since benign tumors proliferate more slowly it is possible that their life span is longer. To test this point the experiment of Berenblum and Shubik should be repeated allowing a greater period of rest before the irritant is applied.

Summary. The influence of interrupted carcinogenic treatment on tumor formation was tested. Rest periods of from one to 3 months were interspersed between 2 periods during which the carcinogens were applied to mice at minimal carcinogenic levels. The investigation was divided into two parts on the basis of the carcinogens employed. In one, ultraviolet irradiation was used for both the first and second periods. In the other, methylcholanthrene was employed to initiate the process and croton oil, a non-carcinogenic substance, was used to evoke the neoplasms. The results with both technics were essentially the same.

A lag in tumor appearance was observed during the rest periods, and the second period of treatment quickly brought forth tumors in the precarcinogenic areas. Essentially no difference in the rate of tumor formation or in the final tumor incidence was observed when the rest period was of one month duration. When the rest period was increased to 3 months, the final tumor incidence was reduced by 34% in the mice irradiated and by 42% in those in the methylcholanthrene-croton oil series. The results indicate that the initial application of the carcinogen produced alterations in the tissues which were maintained for at least one month, thereafter the changes re-

¹¹ Blum, H. F., *J. Nat. Cancer Inst.*, 1943, 3, 569.

¹² Blum, H. F., *J. Nat. Cancer Inst.*, 1944, 4, 559.

Effect of Rutin on Dicoumarolism.*

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As far as the writer is aware, no drug has been described which exerts a specific antagonistic action on the hemorrhagic symptoms of dicoumarolism.

Dicoumarol has been shown to increase bleeding time and this is not correlated with increased prothrombin time.^{1,2} Capillary fragility, moreover, is not altered in patients with hemorrhagic symptoms after excessive dicoumarol.^{2,3} Dicoumarolism also causes marked dilation of capillaries, small arteries and veins.^{4,5} Other toxic vascular changes include marked swelling and vacuolization of endothelium, with areas of endothelium proliferation, degeneration of vascular smooth muscle, perivascular serous exudation and diapedesis of blood cell elements and even necrosis and rupture of the entire vessel wall.

Flavonoid derivatives ("vitamin P"-like substances) have been shown to decrease bleeding time,^{6,7} capillary permeability and fragility. Rutin is a well-known flavonoid described as having anti-capillary fragility action by Sevin,⁸ later by Griffith, Couch and Lindauer⁹ and since then by many others. Widespread

clinical application of rutin has resulted, with conflicting reports. An extensive literature exists¹⁰ on the purported anti-hemorrhagic properties of flavonoids and related substances in diverse pathologic states, both experimental and clinical. The mechanism of such action, if true, is not clear, but may be related to either reduction in the size of the capillary bed,¹¹ or to an antihyaluronidase action.¹²⁻¹⁵

Prandoni and Wright^{2,3} noted no effect of "vitamin P" in the form of grated orange peels orally administered to patients suffering hemorrhagic symptoms from dicoumarol therapy. The quantity of flavonoids administered by this method is small and the nature of the flavonoids contained in orange peel is largely unknown other than hesperidin and eriodictyol.

Naghski, Copley and Couch¹⁶ reported that the flavonol glycosides, rutin and quercitrin, and quercetin (aglucone of rutin) exert an antagonistic effect against the bacteriostatic effect of dicoumarol and therefore suggested the possibility of using rutin or similar flavonols to antagonize the hemorrhagic action of dicoumarol *in vivo*.

The present note presents evidence which indicates that rutin has no protective action against dicoumarolism in rats.

Experimental. Male albino rats (Slonaker

* Supported by a grant from the United States Public Health Service.

¹ Lalic, J. J., Lalic, M. H., and Copley, A. L., *Surgery*, 1943, **13**, 316.

² Wright, I. S., and Prandoni, A., *J. Am. Med. Assn.*, 1942, **120**, 1015.

³ Prandoni, A., and Wright, I., *Bull. N. Y. Acad. Med.*, 1942, **18**, 433.

⁴ Bingham, J. B., Meyer, O. O., and Pohle, F. J., *Am. J. Med. Sci.*, 1941, **202**, 593.

⁵ McCarter, J. C., Bingham, J. B., and Meyer, O. O., *Am. J. Path.*, 1944, **20**, 651.

⁶ Parrot, J., and Galmiche, P., *Compt. rend. Soc. biol.*, 1945, **139**, 948.

⁷ Ungar, G., *Endocrinol.*, 1945, **37**, 329.

⁸ Sevin, A., *Compt. rend. Acad. sci.*, 1943, **216**, 505.

⁹ Griffith, J. G., Jr., Couch, J. F., and Lindauer, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 228.

¹⁰ Anon., "Bibliography of Vitamin P" (256 refs.), *Nutrition Research*, 1945, **5**, No. 1 and 2, and supplements thereof.

¹¹ Fuhrman, F. A., and Crismon, J. M., *J. Clin. Invest.*, 1948, **27**, 364.

¹² Beiler, J. M., and Martin, G. J., *J. Biol. Chem.*, 1947, **171**, 507.

¹³ *ibid.*, 1948, **31**, 174.

¹⁴ Cella, C. J., Jr., and Means, J. A., *Marquette Med. Rev.*, 1948, **13**, 67.

¹⁵ Clark, William G., Unpublished data.

¹⁶ Naghski, J., Copley, M. J., and Couch, J. F., *Science*, 1947, **105**, 125.

TABLE II.
Results of Agglutination Tests Using *Salmonella California* "H" Serum.

Agglutination (after absorption) with "H" antigen from	Serum absorbed with "H" antigen from							
	None	<i>S. californica</i> g, m, t	<i>S. oranienburg</i> m, t	<i>S. essen</i> g, m	<i>S. senftenberg</i> g, s, t	<i>V. comma</i>		
						"k"	"4"	"8"
<i>S. californica</i> , g,m,t	120*	0	20	5	20	80	80	80
<i>S. oranienburg</i> , m,t	60	0	0	5	10	60	60	60
<i>S. essen</i> , g,m	40	0	20	0	5	20	25	20
<i>S. senftenberg</i> , g,s,t	30	0	10	5	0	10	12	10
<i>V. comma</i> "k"	6	0	5	0	0	0	0	0
<i>V. comma</i> "4"	4	0	4	0	0	0	0	0
<i>V. comma</i> "8"	6	0	6	0	0	0	0	0

* All agglutination titers to be multiplied by 100.

TABLE III.
Average of Results of Agglutination Tests Using *Vibrio comma* "OH" Sera.

Agglutination (after absorption) with antigen from	Serum absorbed with antigen from							
	None	<i>S. bredeney</i> I, IV, XXVII, XII	<i>S. pullorum</i> IX, XII	<i>S. worthington</i> I, III, XXIII	<i>S. californica</i> g,m,t	<i>S. oranienburg</i> m,t	<i>S. essen</i> g,m	<i>S. senftenberg</i> g,s,t
<i>V. comma</i> "k"	14*	8	10	10	8	14	9	9
<i>V. comma</i> "4"	12	8	9	9	8	12	8	8
<i>V. comma</i> "8"	12	8	9	9	7	12	7	8
<i>S. bredeney</i> I, IV, XXVII, XII	2	0	1	1	12	2	12	12
<i>S. pullorum</i> IX, XII	2	0	0	2	12	2	12	12
<i>S. worthington</i> I, III, XXIII	2	0	2	0	12	2	12	12
<i>S. californica</i> g,m,t	4	4	4	4	0	4	0	0
<i>S. oranienburg</i> m,t	0	0	0	0	0	0	0	0
<i>S. essen</i> g,m	4	4	4	4	0	4	0	0
<i>S. senftenberg</i> g,s,t	4	4	4	4	0	4	0	0

After absorption with *V. comma* antigens, all agglutination tests were negative.

* All agglutination titers to be multiplied by 100.

sera as high as 5 to 25% of their titer against the homologous *Salmonella* when the serum contained agglutinins for the *Salmonella* antigenic factors I, XII or g. Subsequent absorption tests, as represented in Tables I and II confirmed that parts of *Salmonella* I, XII and g antigens were present in the *V. comma* strains.

Sera prepared against the *V. comma* strains agglutinated *Salmonellae* which contained the antigenic factors I, XII and g. Results of the absorption tests are given in Table III.

Summary. Three Inaba strains of *V. comma* isolated recently in Egypt harbored fractions of *Salmonella* I, XII and g antigens.

Isolation of Mumps Virus from the Blood of a Patient.

LAWRENCE KILHAM. (Introduced by J. F. Enders.)

From the Department of Epidemiology, Harvard School of Public Health, Boston.

The isolation of mumps virus from the blood of a patient is not known to have been reported. One case is described, the methods of isolation and identification being essentially those employed by others for saliva¹ and for spinal fluid.²

Case History. B.M., a boy aged 3 years, 9 months, was admitted to the Children's Hospital of Boston* May 9, 1948, for treatment of cellulitis of the left chest wall incident to a fall. He appeared acutely ill. The temperature was 103°F, an area over the left chest wall was tender and red, and a scarlatiniform rash, thought to be a drug eruption, covered the entire body. Sulfadiazine and penicillin had been administered. Three days later, May 12, the left parotid was firm, swollen and tender. A clinical diagnosis of mumps was made, the temperature at that time being 101°F, pulse 120, and respirations 25. The total leucocyte count on May 11, was 13,800, with 92% granulocytes and 8% lymphocytes; on May 13, it was 10,400, with 52% granulocytes, 38% lymphocytes, and 10% monocytes. Spinal fluid taken May 12 had a trace of globulin, sugar content within normal limits, and no white blood cells. A tuberculin test was positive in 1:1000 dilution of O.T. A marked right parotid swelling appeared on May 16. No signs or symptoms of meningo-encephalitis were noted. Recovery was rapid and the patient returned home May 22.

Isolation of Mumps Virus. Specimens examined for mumps virus were introduced into the amniotic sac of 7-day-old embryonated eggs and amniotic membranes and fluids har-

vested separately after 6 days incubation at 35°C. Fluid from each egg was examined for virus by macroscopic hemagglutination of hen erythrocytes. Cultures in thioglycollate broth of pooled amniotic fluids from each egg passage showed no bacterial contaminants. The amniotic membranes of egg passages giving no hemagglutination were ground with alundum, pooled with their fluids and passed in 0.2 cc amounts intra-amniotically. Only positive fluids were pooled and passed when hemagglutination appeared in subsequent passages. Positive amniotic fluids had a bluish opalescence, those showing no hemagglutination were usually clear.

Blood was drawn from patient B.M. on May 12, the day parotitis was first observed and 5 cc treated with sodium citrate to give a final concentration of 0.5%. Because whole citrated blood had resulted in a high mortality in previous egg inoculations, the plasma was separated from the red blood cells after natural sedimentation, and the two fractions stored in carbon dioxide ice. Two weeks later 12 eggs were inoculated intra-amniotically with 0.1 cc of the plasma, and 5 survived for harvesting. The red blood cell fraction was diluted 1:2 in infusion broth, and the mixture in 0.2 cc amounts inoculated into 12 eggs intra-amniotically. Only 3 embryos survived. Since the amount of harvested material was small, it was added to the amniotic membrane and fluid pool of the plasma passage. In the third egg passage, virus was detected in 4 of 8 amniotic fluids, and in almost all eggs of 2 subsequent passages.

The cerebro-spinal fluid of May 12, inoculated into eggs in 0.2 cc amounts, yielded no virus in 3 passages; a not unexpected result since the patient had no symptoms suggesting meningo-encephalitis and the spinal fluid contained no leucocytes. Isolation of virus from the saliva was not attempted because of the age and lack of cooperation of the patient.

¹ Leymaster, G. R., and Ward, T. G., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 346.

² Henle, G., and McDougall, C. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 209.

* Acknowledgment is made of the kind cooperation and assistance of the hospital staff; also to the Haynes Memorial Hospital for other materials.

strain) were divided into 2 groups of 10 each, averaging 137 g body weight and 2 months of age. Both groups were fed Purina Laboratory Chow mash in which was incorporated 0.04% dicoumarol.[†] One group received this diet alone and the other received the same diet containing 2% rutin[‡] in addition to the dicoumarol. From the average daily food intake it was estimated that each rat ingested about 0.3 mg dicoumarol daily, and the rutin-fed animals ingested about 150 mg rutin daily. Later, as the animals became poisoned, less food was consumed. Records were kept of the hemorrhagic symptoms, body weight loss and survival.

The experiment was terminated after 30 days, at which time there were no survivors in the group fed dicoumarol alone, with an average survival time of 15 days (range 7 to 19); and 10% surviving in the group fed rutin + dicoumarol, with an average survival time of 13 days (range 5 to 21), of the rats which died.

In a second experiment of 10 rats per group, average body weight 120 g, the dietary dicoumarol was adjusted to 0.02% instead of 0.04% as in the first experiment, and the rutin, as before, remained at 2%. The experiment was terminated after 45 days, at which time 90% of the controls survived (the one death occurred in 45 days), and 60% of the rutin-fed animals survived (the 4 deaths oc-

curred in 7, 22, 41 and 44 days).

In a third experiment of 10 rats each, average body weight 140 g, the animals were fed Purina Chow and the dicoumarol \pm rutin were stomach-tubed daily as aqueous suspensions in doses of 5 mg dicoumarol \pm 50 mg rutin. After a week, the dose of dicoumarol was increased to 10 mg, since 5 mg seemed insufficient to evoke hemorrhagic symptoms. The experiment was terminated in 35 days, at which time 30% of the controls were surviving, with an average survival time of 26 days for the rats which died; and 20% of the rutin-fed animals were surviving, with an average survival time of 18 days for the rats which died.

There were no group differences in onset or severity of hemorrhagic symptoms in the 3 experiments.

Conclusions. Under the experimental conditions employed, rutin does not antagonize dicoumarolism in rats. Presumably the defect in the vessel walls or their supporting structures which leads to hemorrhage in dicoumarolism differs from that leading to the various hemorrhagic conditions which rutin and related "vitamin P"-like substances purportedly benefit. These facts, however, need not necessarily preclude the simultaneous use of dicoumarol and "vitamin P" for their separate effects, as illustrated by the report of MacLean and Brabel¹⁷ on the favorable effects of rutin and dicoumarol in various retinal vascular disorders.

[†] Kindly supplied by E. R. Squibb & Sons, New York.

[‡] Kindly supplied by the S. B. Penick Co., New York.

¹⁷ MacLean, A. L., and Brambel, C. E., *Trans. Am. Ophth. Soc.*, 1946, **44**, 194.

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¹ Leymaster, G. R., and Ward, T. G., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 346.

² Henle, G., and McDougall, C. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 209.

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Attempts to isolate mumps virus from the blood of 7 other patients in the first 5 days of the disease were unsuccessful, all but one of the specimens being drawn after the first day of illness. Whole defibrinated blood is less lethal to chick embryo than citrated blood.

Serologic Studies and Identification of Virus. The inhibition of hemagglutination and the complement fixation tests used in identification of mumps virus have been well-described by others.³ Paired serums from a patient, taken in the acute and convalescent phases of mumps and known to demonstrate an increasing titer of inhibiting antibodies, were kindly furnished by Dr. J. F. Enders. Strain B.M. mumps virus, freshly isolated from the blood and used as an antigen with the standard acute serum gave inhibition of hemagglutination in a titer of 1:16; with the convalescent serum in a titer of 1:256, a 16-fold rise, titers being expressed in terms of initial dilution. The clinical diagnosis of mumps for patient B. M. was confirmed by a similar test, using the Enders strain of mumps virus as antigen against paired serums of the patient.† Serum of May 12 had a titer of 1:32 and that of May 22, 1:256, an 8-fold rise in titer.

Identification of the virus as that of mumps was further substantiated by test for fixation of complement with pooled amniotic fluids of the sixth egg passage as antigen. Paired sera from a patient with mumps, and with demonstrated increasing complement fixing antibodies (Diagnostic Laboratory, Massachu-

setts Department of Public Health) were used as a standard. The acute phase serum had a titer of 1:16 and the convalescent serum 1:256.

A complement fixation test of serums from patient B. M., using the Enders strain of mumps virus as antigen, gave a titer of 1:32 with acute phase serum and a four-fold rise to 1:128 with convalescent serum.

Isolations from Spinal Fluid. Successful isolation of mumps virus has been accomplished in 5 or 7 cases of meningo-encephalitis, all of which demonstrated a rise in complement fixing antibodies during convalescence, where spinal fluids were examined within 5 days of onset. An 8-fold or greater rise of titer in the inhibition of hemagglutination test was used in identification of the viruses isolated.

Discussion. A virus was isolated from the blood of a patient on the first day of a parotid swelling conforming to that of mumps. The clinical diagnosis was confirmed by a significant rise in titer of mumps complement-fixing and inhibition of hemagglutination antibodies in the course of convalescence. Two methods, inhibition of hemagglutination and complement fixation, identified the virus as that of mumps.

That mumps virus invades the blood stream is not surprising, in view of the considerable amount of virus apparently present in an infected parotid gland. The regularity of such invasion and its significance in the pathogenesis of the disease, awaits the result of studies now in progress.

Summary. The virus of mumps was isolated from the blood of a patient with bilateral parotid swelling through culture in embryonated eggs. Inhibition of hemagglutination and complement fixation tests confirmed the clinical diagnosis and identified the agent as the mumps virus.

³ Diag. Procedures for Virus and Rickettsial Diseases. Am. Pub. Health Assn., 1948, p. 139.

† Test was kindly performed by the Research Division of Infectious Diseases, Children's Hospital, Boston.

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Relative Lack of Myelin in Optic Tracts as Result of Underfeeding in the Young Albino Rat.*

A. R. BUCHANAN AND J. E. ROBERTS.

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The underfeeding experiments reported herein were originally started, and are still being carried on, to determine the effects of inanition on the ability to regulate body-temperature. We have previously presented experimental evidence indicating that normal myelination of hypothalamic fibers of young rats may have an important bearing upon the attainment of regulatory ability.¹ The myelin lack in the optic tracts of underfed animals has been an incidental finding, but one which may have considerable practical importance.

Donaldson² studied the effects of underfeeding on myelination in the central nervous system of the albino rat. His rats were 30 days old at the beginning of the experiment and 51 days old when sacrificed. The average percentage difference in body weight, at the end of the experiment, between his control and underfed animals was 41.2, and the average percentage difference in brain weight was 7.7. Weigert-stained sections did not show grossly observable differences between the myelin content of the brains from the control and those from underfed rats. Donaldson concluded from these studies that underfeeding does not arrest myelination.

Methods and Materials. Six litters of Wistar strain albino rats have been used in the present investigation. On the 17th to 20th day of life, 3 rats from each litter were weaned, and placed in individual cages, where each was maintained on a diet of 5 g of Purina laboratory chow per day. The control animals were left with their mothers until they had reached 26 days of age, when

they were weaned and then maintained on Purina laboratory chow *ad libitum*. The underfed animals and their litter-mate controls were sacrificed on the 50th to the 52nd day of life.

When sacrificed, the experimental and control animals were perfused with Regaud's solution (3½% potassium dichromate—80 parts; neutral formalin—20 parts). Following perfusion, the brains were removed, weighed, and placed in 5% potassium dichromate. They were left in this solution for 10 days at 38-40°C; the solution was renewed on the 2nd, 5th and 8th days. When all the brains from a given litter had been embedded in paraffin, they were sectioned at 20 micra and mounted on slides. Representative slides from all the animals in the litter were placed together in a staining rack and stained en masse according to the method reported by Ulett, Dow and Larsell.³

The concentration of myelin in the optic tract was determined on the basis of monochromatic light absorption by stained sections as previously described.¹ After focusing upon the optic tract the slide was moved to an adjacent area which was free of sections and, without altering this focal adjustment, the amount of light entering the microscope was adjusted by means of a rheostat and the iris diaphragm at the inlet of the monochromator to give a reading of 80.0 on the galvanometer of the electrophotometer. The light was adjusted to this same "zero" reading in relation to each of the determinations in a given series, and this compensated for differences in thickness of slides, cover slips, and mounting media. When the light had been so adjusted, the optic tract was moved back into the field and the galvanometer reading record-

* Supported by a grant from the Office of Naval Research.

¹ Buchanan, A. R., and Hill, R. M., *Proc. Soc. Exp. Biol. and Med.*, 1947, 66, 602.

² Donaldson, H. H., *J. Comp. Neurol.*, 1911, 21, 139.

³ Ulett, G., Dow, R. S., and Larsell, O., *J. Comp. Neurol.*, 1944, 80, 1.

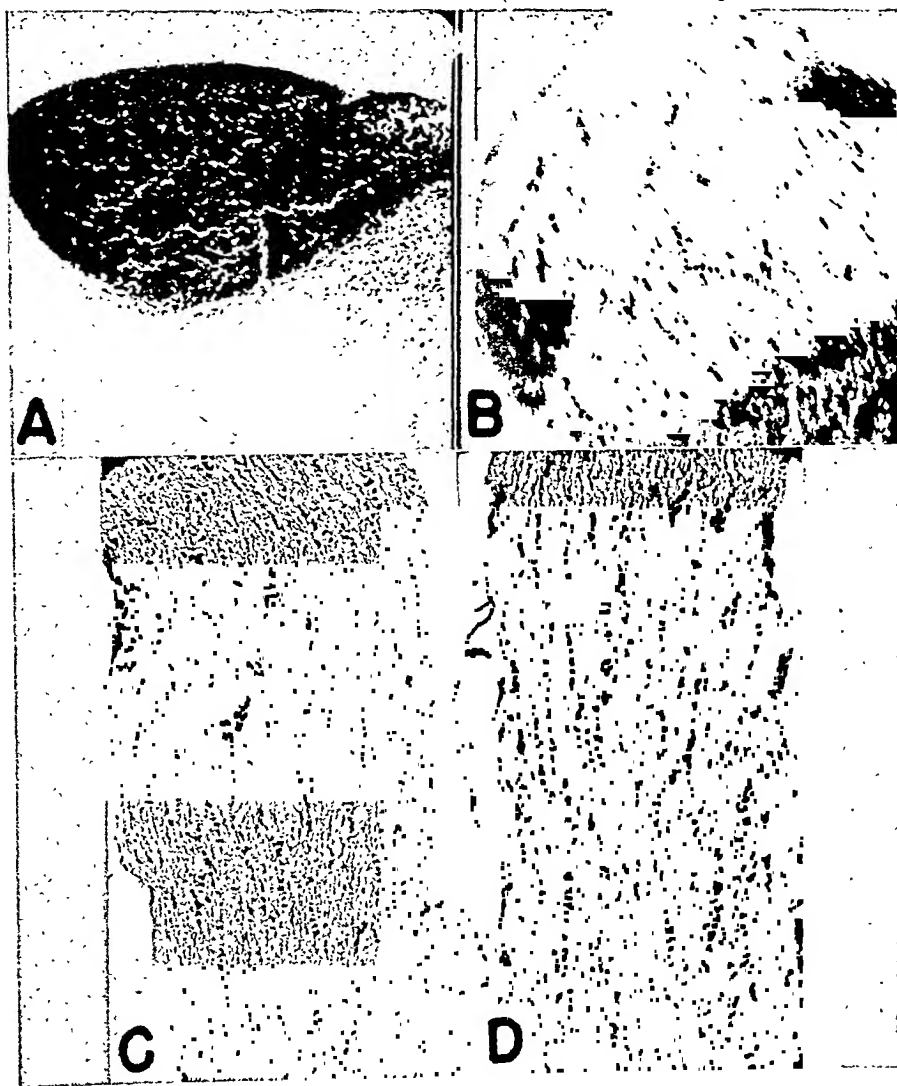


FIG. 1.

A. Optic tract from rat 1-F-3 (exp.); B. Optic tract from rat 1-F-4 (control); C. Optic nerve from rat 2-O-3 (exp.); D. Optic nerve from rat 2-O-6 (control). The optic tracts were stained for myelin by the method of Ulett *et al.*, and the optic nerves were stained by the method of Weil.

ed. The difference between this reading and the zero reading represented the amount of light absorbed by a given section. This procedure was carried out on 6 sections from a given animal. One hundred times optical density was computed for each reading as a measure of relative myelin concentration. The optical density here used was the log of the ratio of incident (galvanometer reading of 80.0) to ensuing light intensity in accordance

with the Beer-Lambert law of absorption.

Results. A relative lack of myelin in the optic tracts of underfed rats, as compared to that present in those of litter-mate controls, appeared in the majority of cases. In many instances the difference was of sufficient magnitude to be apparent without the use of the electrophotometer. This is demonstrated by the photomicrographs presented in Fig. 1, in which the optic tracts from Rat 3 (ex-

TABLE I.
Summary and Statistical Evaluation of Results.

Litter	Myelin (experimental)	Myelin (control)	% difference	Significance	% difference body wt	% difference brain wt
Q	35.6 37.6 41.0	35.9 43.3 45.0	8.0	P = 1 (approx.)	28.6	13.5
S	29.9 38.2 28.6	41.7 43.6 25.4	12.6	P = 1 (approx.)	64.9	22.0
Y	42.6 35.1 45.3	50.2 55.2 47.9	19.8	P = 0.05	70.7	21.0
1-F	60.6 32.4 37.4	63.8 55.9 60.4	27.7	P = 0.15 (approx.)	57.6	14.9
2-K	39.8 31.3 33.3	46.4 40.5 37.2	15.9	P = 0.16 (approx.)	60.7	17.5
2-O	29.2 36.7 27.0	52.2 43.6 46.2	34.5	0.05 > P > .01	48.3	13.5
Means	(36.8)	(46.4)	19.8	P < 0.01	55.1	17.1

perimental) and Rat 4 (control) of litter 1-F are illustrated (A and B) together with the optic nerves from Rat 3 (experimental) and Rat 6 (control) of litter 2-0 (C and D). The optic nerves were not studied routinely since, in those cases in which they were, those from underfed rats showed the same relative lack of myelin as was apparent in the tracts of the same animals.

Gross examination of the optic foramina of the underfed animals has failed to show any evidence that the amyelination observed in their optic nerves and tracts is due to stenosis of the foramina such as has been described by Moore⁴ in carotene-deficient calves.

Statistical analytical methods have been applied to our data by one of us (J.E.R.) and have served to show that the spectrophotometric method is reliable and that the results have statistical significance.

The average values of 6 determinations of relative myelin concentration (100 x optical density) for each underfed and control animal within the respective litters are shown

in Table I. The percentage differences given in the table are based upon differences of means between underfed and control rats, relative to the mean of the controls. The succeeding column gives the approximate significance of this difference for each litter based upon analysis of variance within the respective litters. The last 2 columns give average per cent decrease in brain weight and body weight for each litter to indicate relative magnitude of inanition.

An analysis of variance was applied to the data of each litter to determine the sources of variation (1) between the myelin concentrations of underfed and control animals, (2) among the original determinations of myelin concentration, and (3) among the myelin concentrations of underfed and starved animals of each litter. The experimental errors (differences in section thickness, staining, spectrophotometric readings, etc.) were negligible when compared with the two other sources of variation in the experiment, since mean squares ranged from 2 to 7 in all litters except 2-K, where the value was 21. There was, in general, considerable variation between myelin concentrations among animals within

⁴ Moore, Lane A., *J. Nutrition*, 1939, 17, (Supp.), 10.

the control and experimental groups of each litter; mean squares ranged from 92 to 193 in all litters except 1-F, where the value was 725.

Comparison of the variance between underfed and control groups, where mean squares ranged from 98 to 2475, with the variance among animals within the groups becomes the critical factor of the analysis within litters. The F-test gave indications of significance as shown in the table. By this test the differences in myelin concentration could have occurred by chance within litters Q and S due to large variance among animals of experimental and control groups, are of moderate degree of significance in litters 1-F and 2-K, and are highly significant in litters Y and 2-O.

In determining the significance of underfeeding on optic tract myelination in all 6 litters, 2 major sources of variation, namely, differences in staining between litters and inherent differences in myelin content between litters, were not segregated experimentally. While such inherent variations between litters would be of considerable interest, it appears, from the standpoint of determining the effect of underfeeding on myelination, that the factor of greatest importance is the significance of the differences in myelin density that appeared in the entire group of 6 litters. An F-test comparing total variance between underfed and control animals of each litter with the total variance within litters gave high significance to the difference in myelin ($P < 0.01$). Thus, for the total experiment, there seems to be a significant difference of myelin concentration between control and underfed rats of the order of magnitude of 20%.

Discussion. The relative lack of myelin in the optic nerves and tracts of the majority of our underfed rats, determined photometrically, must be considered as indicative of partial or complete arrest of the process of myelination. Curves constructed from photometric determinations of myelin densities in the optic tracts of successively older members of several litters of albino rats indicate that myelination within the visual pathway continues to be an active process until sometime between the 40th and 50th days as compared

with its completion in the medullary pyramid and hypothalamus between the 18th and 30th days.¹ This delay in myelination of the components of the visual pathway may be due to a lack of functional stimulation until after the eyes have opened (14th to 17th day). Tilney and Casamajor² reported that they were able to correlate beginning myelination of the optic nerves and the eye-opening reaction in kittens.

The accuracy of the results reported herein depends upon the assumption that the myelin concentration is directly related to optical density in accordance with the Beer-Lambert Law of Absorption. The use of monochromatic light seems to validate this assumption. Whereas slight deviation from the law may be present, the differences found for the entire group of 6 litters appear to be of sufficient magnitude and reliability that such deviation should not alter the general conclusions.

Summary and Conclusions. Quantitative limitation of food intake sufficient to account for average body-weight differences of 55% between underfed and litter-mate control rats, has also resulted in statistically significant differences in myelination of the optic tracts and nerves. The concentration of myelin in the optic tracts of the underfed rats has averaged 20% less than that in the control animals. Since myelination of the optic tracts, as shown by spectrophotometric determinations in litter-mate rats at successively older ages, is not complete until sometime between the 40th and 50th days, the conclusion seems justified that the relative lack of myelin observed in the underfed animals is due to partial or complete arrest of the process of myelination.

The authors wish to thank Mr. W. S. Worley, Supervisor of Special Projects, Product Application Department, Gates Rubber Company, for valuable suggestions and criticisms in regard to the statistical analysis and to acknowledge the painstaking care with which the photomicrographs were prepared by Mr. Glenn Mills.

¹ Tilney, F., and Casamajor, L., *Arch. Neur. and Psychiat.*, 1924, 12, 1.

16631

Experimental Streptomycin Therapy in Mice.*†

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In contrast to the sulfonamides, penicillin and streptomycin do not require constant significant blood levels for satisfactory therapeutic results. Experiments in streptococcal infections of mice¹ have clearly indicated that the effect of crude penicillin on the bacterial population outlasts measurable blood levels by many hours. Animals were cured of otherwise fatal infections with single large doses or by spacing injections of penicillin 8-24 hours apart.^{2,3,4} Clinical experience in pneumococcal^{5,6} and streptococcal⁷ disease has also demonstrated the effectiveness of dosage regimens by which aqueous penicillin was injected every 8-12 hours. Experiments with streptomycin have given comparable results.⁸

A possible objection to some of the experimental work presented in this field and its translation into clinical terms is the nature of the laboratory model often used for evaluation of chemotherapeutic effectiveness. Animals are given overwhelming infections, usually by "unnatural" routes, and then treated before there is clinical evidence of the disease. The results thus present the effects of chemoprophylaxis rather than chemotherapy.

In the course of studies on a pneumotropic pasteurella of mice⁹ observations were made which are of some interest in regard to this question.

Methods. The pneumotropic pasteurella used in this work has been described elsewhere.⁹ The organism kills mice only if inoculated intracerebrally (LD₅₀: ca. 10⁴ bacteria) or intranasally (LD₅₀: ca. 5×10⁵ bacteria). After intracerebral inoculation neurological signs appear within 12-18 hours and all deaths occur in 36-72 hours with a brain abscess. When infected by the respiratory route the mice show cyanosis and dyspnea, with pulmonary consolidation in 18-24 hours and 70-95% die in 2-6 days. The pasteurella is sensitive to streptomycin. It is regularly completely inhibited by 2-4 γ of streptomycin per milliliter of medium.

The standard inoculum in these experiments consisted of 10⁶ bacteria in 0.03 ml of dilutions of a 8-12-hour broth culture, injected intracerebrally or intranasally under light ether anesthesia into 21-day-old (10-14 g) white mice of the stock of the National Institute of Health. The animals were observed for 10 days and deaths recorded. At the end of that period the survivors were autopsied and lung lesions were noted. Among the intracerebrally infected animals there were no untreated survivors, and the treated survivors showed no lesions whatever. The results of these treatment experiments are recorded as the ratio of deaths to the total number of animals in the group. Among intranasally infected animals, however, not all untreated mice died and some of the treated survivors showed extensive lung lesions. In these groups the results are therefore expressed as ratio of "infected" animals to the total number in the group. "Infected"

* The work described here was performed at the Division of Infectious Diseases, National Institute of Health, Bethesda, Md.

† The author wishes to thank Doctors M. Finland and E. K. Marshall, who read the manuscripts and made valuable suggestions.

¹ Jawetz, E., *Arch. Int. Med.*, 1946, **77**, 1.

² Zubrod, C. G., *Bull. Johns Hopkins Hosp.*, 1947, **81**, 400.

³ Eagle, H., *Am. Int. Med.*, 1948, **28**, 260.

⁴ White, H. J., Baker, M. J., and Jackson, E. R., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 199.

⁵ Tillett, W. S., Cambier, M. J., and McCormack, J. E., *Bull. N. Y. Acad. Med.*, 1944, **20**, 142.

⁶ Marshall, E. K., and Zubrod, C. G., Personal communication.

⁷ Jawetz, E., *Arch. Int. Med.*, 1948, in press.

⁸ Zubrod, C. G., *Bull. Johns Hopkins Hosp.*, 1948, **82**, 357.

⁹ Jawetz, E., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 46.

TABLE I.

Streptomycin Treatment of Intracerebral Pasteurella Infection.

Mice infected with 100 LD₅₀ intracerebrally given 3 subcutaneous injections of 0.1 g/kg streptomycin each, at various intervals.

Treatment time, hr after infection	Deaths/total	% survivors (cured)
2, 6, 24	2/12	83.3
2, 24, 48	1/12	91.6
6, 10, 24	6/12	50.0
6, 24, 48	8/12	33.3
10, 24, 48	12/12	0.0
Controls (untreated)	24/24	0.0

refers to both the dead animals and those which survived but had gross lesions in the lungs.

The streptomycin (a solution with an assay value of 200 mg streptomycin base/ml) was kindly supplied by Dr. H. Welch, Food and Drug Administration, Washington, D.C. Solutions of the required strength were prepared in physiological saline and were injected subcutaneously in amounts of 0.2 ml per dose as indicated in the various dosage schedules.

Results. Table I summarizes selected results of preliminary trials in the treatment of intracerebral infections by subcutaneously administered streptomycin. With fixed individual and total dose the outstanding factor which determined success of therapy was the interval between the injection of bacteria and the first administration of streptomycin. It was immaterial whether the second dose was given 4 or 22 hours after the first. If the total dose exceeded 0.3 g/kg and the first of 3 injections was given not later than 4 hours after intracerebral infection at least 75% of the animals could be cured. For therapy to be successful it had to be started long before neurological symptoms appeared.

When identical treatment schedules were applied to groups of mice infected intracerebrally or intranasally, the milder respiratory infection responded much more readily. With the first dose of antibiotic administered to all mice 4 hours after infection, there was little difference between the results of various dosage schedules (Table II). The ED₅₀ (dose resulting in 50% cure)¹⁰ for cure of infection of the central nervous system ranged

between 71 and 108 mg/kg with the various regimens, and it was approximately half of those values for the pulmonary disease. These results considered by themselves might indicate similar response of the "artificial" (intracerebral) and "natural" (respiratory) infection to streptomycin treatment. It must be considered, however, that treatment 4 hours after infection is relatively early for the latter and late for the former.

Good evidence, relating the time elapsed before treatment was started to the therapeutic results, was obtained in the comparative tests illustrated in Table III. If treatment was started 4 hours after infection, when neither symptoms nor lesions were present, a number of schedules of therapy were satisfactory. There was no marked difference between total doses of 0.05 g/kg and 0.3 g/kg whether administered in a single dose or in three injections 2 or 24 hours apart. If, however, 18 hours were permitted to elapse between infection and first treatment, statistically significant differences appeared between the results of various low-dose schedules. With a total of 0.1 g/kg, the single dose was quite unsatisfactory, whereas with 3 doses administered at 2-hour intervals, only one-fourth of the animals showed lesions. Three injections 24 hours apart gave intermediate results. These differences between various dosage schedules were not apparent at higher total dose levels (Table III). In order to obtain satisfactory response with long-interval regimens, the dosage had to be larger than with schedules employing injections every 2-4 hours.

Comment. Experimental work in chemotherapy is often based on overwhelming types of infection. Small numbers of highly virulent organisms are introduced into animals in such a manner as to permit rapid multiplication and to produce prompt death. Thus the animals represent little more than living test-tubes to which chemotherapeutic agents are added early in the infection, prior to the appearance of symptoms and lesions. Under

¹⁰ Quan, S. F., Foster, L. E., Larson, A., and Meyer, K. F., *Proc. Soc. Exp. Biol. and Med.*, 1948, 66, 528.

TABLE II.
Comparison of Treatment Results with Various Dosage Schedules in Intranasal and Intracerebral Pasteurella Infection.
Treatment Schedules.

Total dose streptomycin g/kg	Intranasal infection				Intracerebral infection			
	Single inj.	3 inj. q 2 h	3 inj. q 8 h	3 inj. q 24 h	Single inj.	3 inj. q 2 h	3 inj. q 8 h	3 inj. q 24 h
.05	9/20*	5/20	4/19	5/20	16/20†	14/20	17/20	15/20
.20	4/30	2/30	3/29	4/30	8/20	5/20	2/20	10/20
.30	4/30	1/28	3/30	3/30	7/20	1/20	1/19	6/20
.40	—	2/20	1/18	1/20	—	0/19	0/20	1/20
0	46/50	—	—	—	60/60	—	—	—

All mice infected with 10⁶ bacteria. First dose of antibiotic given 4 hr after infection.

* Infected/Total.

† Deaths/Total.

TABLE III.
Influence on Therapeutic Results of the Time Elapsed Before Treatment Was Started.
Total Dose of Streptomycin.*

Treatment started	Dosage schedule	0.1 g/kg		0.3 g/kg		ED ₅₀ g/kg	S.E. of ED ₅₀ g/kg
		Inf./Tot.	% lesions	Inf./Tot.	% lesions		
4 hr after infection	Single dose	13/40	32.4	4/10	10.0	.081	± .013
	3 × q2h	8/39	20.5	1/40	2.5	.052	± .009
	3 × q24h	7/40	17.2	3/38	7.9	.063	± .011
18 hr after infection	Single dose	35/40	87.5	12/40	30.0	.219	± .028
	3 × q2h	11/40	27.5	8/40	20.0	.048	± .020
	3 × q24h	19/39	48.6	9/40	22.5	.106	± .014
Controls	0	49/52	94.2	—	—	—	—

All mice infected with 10⁶ pasteurella.

ED₅₀: Dose resulting in 50% freedom from lesions. S.E. of ED₅₀: Standard error.¹⁰

* Only 2 doses are given here. Additional ones were used to calculate ED₅₀ and its standard error.

such conditions the natural defense mechanisms of the host do not commonly come into play and the animals die from intoxication or organ necrosis, often without the development of gradually progressive lesions.

In a few human infections, like pneumonic plague, the pathogenesis parallels that seen in experimental animals,¹¹ and experimental chemotherapeutic results may be directly applicable to patients. In the majority of human infections, however, the disease is much more complex than that of the "test-tube" experiment in animals. Host reactions influence the development of tissue lesions which may have a spontaneous tendency toward healing, and antibiotics probably support mechanism of defense rather than replace

them. In human disease chemotherapy is seldom started prior to the development of symptoms or lesions. The extent to which results of "test-tube" experiments in animals mirror events in patients is debatable.

The observations reported in this paper indicate that route of inoculation, nature of disease, and the time treatment is started, can influence considerably the relative merits of various dosage schedules employed in chemotherapy.

Intracerebral pasteurella infection can be cured by streptomycin, subcutaneously administered to mice. This in itself is a remarkable finding. The data cannot be used to support the opinion¹² that some types of human central nervous system infections like-

¹¹ Litchfield, J. T., and Fertig, J. W., *Bull. Johns Hopkins Hosp.*, 1941, 69, 276.

¹² Hoyne, A. L., and Brown, R. H., *J.A.M.A.*, 1948, 136, 597.

wise are cured by intramuscular streptomycin alone. The intracerebral pasteurella infection of mice is strictly of the "test-tube" type and treatment is successful only if started prior to the appearance of symptoms.

Mice infected with the same pasteurella by the "natural" respiratory route suffer from a slower, progressive disease and die usually from anoxia due to widespread pneumonic lesions. Streptomycin is effective when started relatively late in that disease process, but even high doses may cure only a certain proportion of the animals. Such late treatment emphasized differences between dosage schedules, not apparent under less critical conditions. The use of similar "natural" types of infection in chemotherapeutic testing is not likely to yield as clear-cut pharmacological results as the "test-tube" types but it might contribute information on the dynamics of antibiotic action important in human disease.

Summary. 1. The therapeutic effect of streptomycin was evaluated in mice infected with a pneumotropic pasteurella either by the

respiratory or by the intracerebral route.

2. When treatment was started within 4 hours after infection intracerebrally mice could be saved by streptomycin injected subcutaneously. With a total dose of 0.3 g/kg streptomycin the main factor determining success of therapy was the time interval between infection and first administration of antibiotic.

3. With comparable dosage regimens larger amounts of streptomycin were necessary to cure the intracerebral than the respiratory infection.

4. When treatment of the pulmonary infection was delayed for 18 hours, until symptoms and lesions were present, marked differences appeared in the effectiveness of dosage schedules similar in their effects when treatment was started soon after infection.

5. The possible usefulness of "natural" type infections in chemotherapeutic experiments is discussed and compared to the "test-tube" type of experimental disease.

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Failure of an Extract of Pregnant Mares' Urine to Influence Gastric Secretion in Man.

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The effect of extracts of urine upon gastric secretion and upon the course of experimentally induced ulcer has been studied by many investigators. Sandweiss, Saltzstein, and Farbman^{1,2} noted delay or prevention of ulcers in the dog following the administration of extracts prepared from human pregnancy urine and the urine from normal women. Various observers³⁻⁹ have reported the pres-

ence in human and canine urine of a substance inhibiting gastric motility and the secretion stimulated by histamine and by a meal. Fur-

¹ Sandweiss, D. J., Saltzstein, H. C., and Farbman, A., *Am. J. Dig. Dis.*, 1938, **5**, 24.

² Sandweiss, D. J., Saltzstein, H. C., and Farbman, A., *Am. J. Dig. Dis.*, 1939, **6**, 16.

³ Friedman, M. H. F., Recknagel, R. O., Sandweiss, D. J., and Patterson, T. L., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 509.

⁴ Necheles, H., Hanke, M. E., and Fantl, E., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 618.

⁵ Gray, J. S., Wieczorowski, E., and Ivy, A. C., *Science*, 1939, **89**, 489.

⁶ Culmer, C. U., Atkinson, A. J., and Ivy, A. C., *Endocrinology*, 1939, **24**, 631.

⁷ Gray, J. S., Wieczorowski, E., Culmer, C. U., and Adkinson, J. L., *Am. J. Physiol.*, 1940, **129**, 589.

⁸ Culmer, C. U., Gray, J. S., Adkinson, J. L., and Ivy, A. C., *Science*, 1940, **91**, 147.

⁹ Friedman, M. H. F., and Sandweiss, D. J., *Am. J. Dig. Dis.*, 1941, **8**, 366.

TABLE I.
Effect of Extract of Pregnant Mares' Urine (Kutrol) on Nocturnal Gastric Secretion of Patients with Peptic Ulcer.

		Control period			Amt Kutrol (g)	After period		
	Case	Vol. (cc)	Free HCl (Cl units)	Total output HCl (mg)		Vol. (cc)	Free HCl (Cl units)	Total output HCl (mg)
Active Material								
C.O'K. No. 423401 ♂, 43 gastric ulcer	1	585 440	53 50	1120 801	1.125	590	54 1151	
J.D. No. 324931 ♂, 50 gastric ulcer	2	619 844	20 22	440 662	1.875	1048	38 1434	
K.A. No. 421560 ♀, 44, duod. ulcer	3	919	52	1743	1.875	953	57 1965	
A.B. No. 416714 ♂, 44, duod. ulcer	4	637	18	443	1.875	526	24 470	
E.D. No. 427655 ♂, 38, duod. ulcer	5	1171	64	2704	7.125	922	51 2207	
J.L. No. 214008 ♂, 39, duod. ulcer	6	1326	79	3831	7.125	1252	53 2393	
E.R. No. 428179 ♂, 56, duod. ulcer	7	830	68	2062	7.125	647	57 1332	
S.R. No. 435305 ♂, 52, duod. ulcer	8	1124	81	3314	7.50	1273	76 3456	
Control Material								
J. McA No. 347846 ♂, 33, duod. ulcer	9	1163	47	2028	7.125	1140	48 2415	
A.H. No. 432870 ♀, 56, duod. ulcer	10	918	71	2377	7.125	1262	69 3191	
A.H. No. 403706 ♂, 57, duod. ulcer	11	1135	48	1965	7.125	910	53 1785	
J.R. No. 232510 ♂, 57, duod. ulcer	12	1054	69	2633	7.125	1139	67 2513	

ther study¹⁰⁻¹¹ resulted in a preparation, designated as urogastone, free of pyrogenic impurities and gonadotropic hormones. When administered subcutaneously, the quantity of extract effective against the ulcers resulting from the Mann-Williamson operation appeared too small to reduce gastric secretion; the protective action, therefore, was attributed to some factor other than that inhibiting gastric secretion. Sandweiss *et al.*¹²

¹⁰ Gray, J. S., Culmer, C. U., Wiczorowski, E., and Adkinson, J. L. *Proc. Soc. Exp. Biol. and Med.*, 1940, 43, 225.

¹¹ Gray, J. S., Wiczorowski, E., and Ivy, A. C., *Am. J. Dig. Dis.*, 1940, 7, 513.

observed beneficial effects against experimental ulcer in the presence of an essentially unaltered acid gastric juice; the protective agent was found in the urine of pregnant and of normal females, but only in small quantities in the urine of patients with peptic ulcer.

The latest preparation to receive attention is an extract of the urine from pregnant mares, termed Kutrol. This material is described¹³ as entirely devoid of estrogenic or gonadotropic activity, highly soluble in water, and

¹² Sandweiss, D. J., Sugarman, M. H., Friedman, M. H. F., Saltzstein, H. C., and Farbman, A. A., *Am. J. Dig. Dis.*, 1941, 8, 371.

¹³ Sharp, E. A., Personal communication.

manifesting an acceptable degree of antiulcer activity when assayed on the Shay rat; its precise nature is, however, not known. Bercovitz, Page, and Heffner¹⁴ recently have reported encouraging results in the treatment of chronic duodenal ulcer with doses of 0.6 g by mouth daily in divided amounts. It appeared desirable, therefore, to determine the effect of Kutrol upon gastric secretion in man.

Method of Study. The method of investigation was similar to that described previously.^{15,16} Twelve patients, 10 with active duodenal ulcer and 2 with gastric ulcer, were studied. Continuous gastric suction was maintained by a Gomco aspirator, the containers being replaced each hour. In 4 patients the 12-hour nocturnal gastric secretion was measured before and approximately 2 hours after the administration of the extract* via the stomach tube; one individual received 0.225 g every 2 hours for 5 doses, a total of 1.125 g; the remaining 3 were given 1.875 g in divided amounts. The subjects were maintained on a liquid diet during the interval when aspiration was discontinued.

In 4 patients gastric suction was continued for 36 hours. After 16 or 17 hours (either at 1:30 or 2:30 p.m.) 7.125 to 7.50 g of Kutrol were introduced into the stomach via the Levine tube. Aspiration was resumed after an interval of 3 hours, and maintained for an additional 16 or 17 hours. The remaining 4 patients were studied in an identical manner but received 7.50 g of an "inactive" material; this group, therefore, served as an additional control. Fluid and electrolyte balance was maintained in the two latter groups by the intravenous administration of 5% glucose in isotonic saline solution.

¹⁴ Bercovitz, Z. T., Page, R. C., and Heffner, R. R., *Am. Gastroenterological Assn.*, May 1, 1948, Atlantic City, N. J.

¹⁵ Levin, E., Kirsner, J. B., Palmer, W. L., and Butler, C., *Arch. Surg.*, 1948, **46**, 845.

¹⁶ Kirsner, J. B., Levin, E., and Palmer, W. L., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 90.

* Made available through the kindness of Drs. Z. Bercovitz and E. A. Sharp. The extract was prepared in kapseals, each containing 0.075 g of the active material in the form of a powder.

Results. The data for the 12-hour nocturnal gastric secretion are summarized in Table I. The volume, free acidity and output of hydrochloric acid were not significantly reduced despite the administration of 7.50 g of the extract of pregnant mares' urine, a quantity 12 times larger than the present clinical dose. The control material likewise was without discernible effect. The administration of Kutrol did not decrease the con-

FAILURE OF EXTRACT OF PREGNANT MARES' URINE (KUTROL) TO INHIBIT FASTING GASTRIC SECRETION

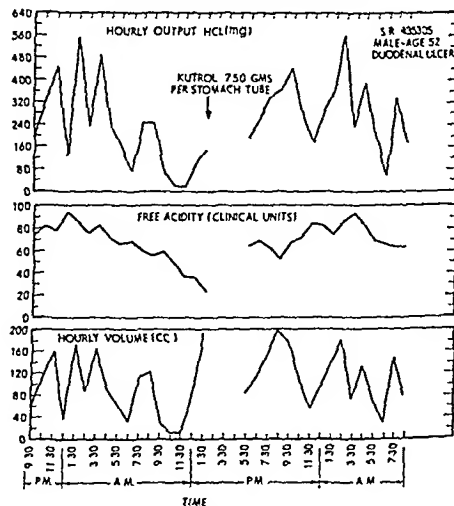


FIG. 1.

FAILURE OF "INACTIVE" EXTRACT OF PREGNANT MARES' URINE (CONTROL) TO INHIBIT FASTING GASTRIC SECRETION
A.N. 432870 FEMALE-AGE 56 DUODENAL ULCER

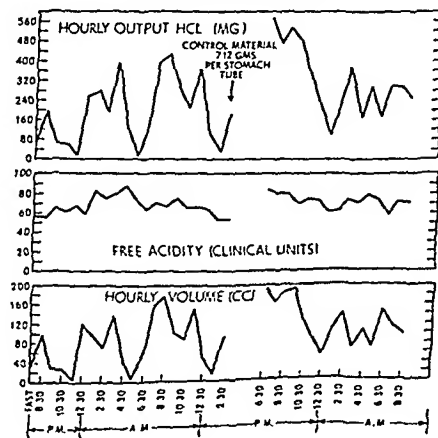


FIG. 2.

tinuous, fasting, 36-hour secretion; representative studies with the active extract and the control material are shown in Fig. 1 and 2. The hourly volume, free acidity, and output of hydrochloric acid remained essentially unchanged. No untoward effects were observed in these studies.

Conclusion. The intragastric administration of an extract of pregnant mares' urine, Kütrol, in quantities as large as 7.50 g does not decrease the volume, free acidity, or output of hydrochloric acid in the 12-hour nocturnal gastric secretion or in the continuous 36-hour secretion of patients with peptic ulcer.

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Effect of Forced Expiration upon Finger and Pulse Volume.*

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Although there is general agreement about the peripheral arteriolar constrictive effect of a deep inspiration, the effect of a deep expiration upon peripheral circulation is not yet clarified. Bolton, Carmichael, and Sturup¹ and Gilliat² report no effect on finger volume, whereas Uhlenbruck³ and de Lalla⁴ observed a diminution of finger volume, and the latter reported coincident decrease in skin temperature and arterial rate of flow following a forced expiration. Therefore it seemed of interest to study further the possible changes in finger and pulse volume incident to a deep expiration.

Method. Pulse and finger volumes were recorded in two ways using a finger plethysmograph each time: (1) In 11 cases the plethysmograph changes were picked up by a piezo-electric crystal whose output was fed into an amplifier-galvanometer recording system. The deflections of the mirror galvanometer were recorded on film moving at

25 mm per second; in 4 of these tracings a simultaneous ballistocardiogram was recorded. With this method any desired amplification of the pulse volume could be obtained, but the total finger volume changes could not be recorded since the system would not maintain a change of baseline; (2) In 5 cases the output of the plethysmograph was put directly into a Frank capsule whose mirror deflections were recorded in the same manner as above. Although the pulse impulse waves were not large in amplitude with this technic, the total finger volume changes were accurately recorded. Respirations were recorded simultaneously in all cases by a pneumograph-Frank capsule arrangement.

Six normal adult subjects were used, 4 males and 2 females, and a total of 16 records were obtained. They were instructed in the following manner: "At no time take a deep inspiration; at the end of a normal inspiration expire forcefully and rapidly, as much as you can, through your mouth; do not hold your breath, but continue to breathe normally directly afterwards." This maneuver is described in detail since the manner of performance seems to be important.

Results. In all cases there was an immediate decrease in pulse volume, and in the 5 tracings recorded with the Frank capsule there was a simultaneous fall in total finger

* This work was carried out under a contract between the Office of Naval Research and the University of Rochester, School of Medicine and Dentistry.

¹ Bolton, B., Carmichael, E. A., and Sturup, G., *J. Phys.*, 1946, **80**, 83.

² Gilliat, R. W., *J. Physiol.*, 1948, **107**, 76.

³ Uhlenbruck, P., *Z. Biol.*, 1924, **80**, 317.

⁴ de Lalla, V., *Am. J. Physiol.*, 1948, **152**, 122.

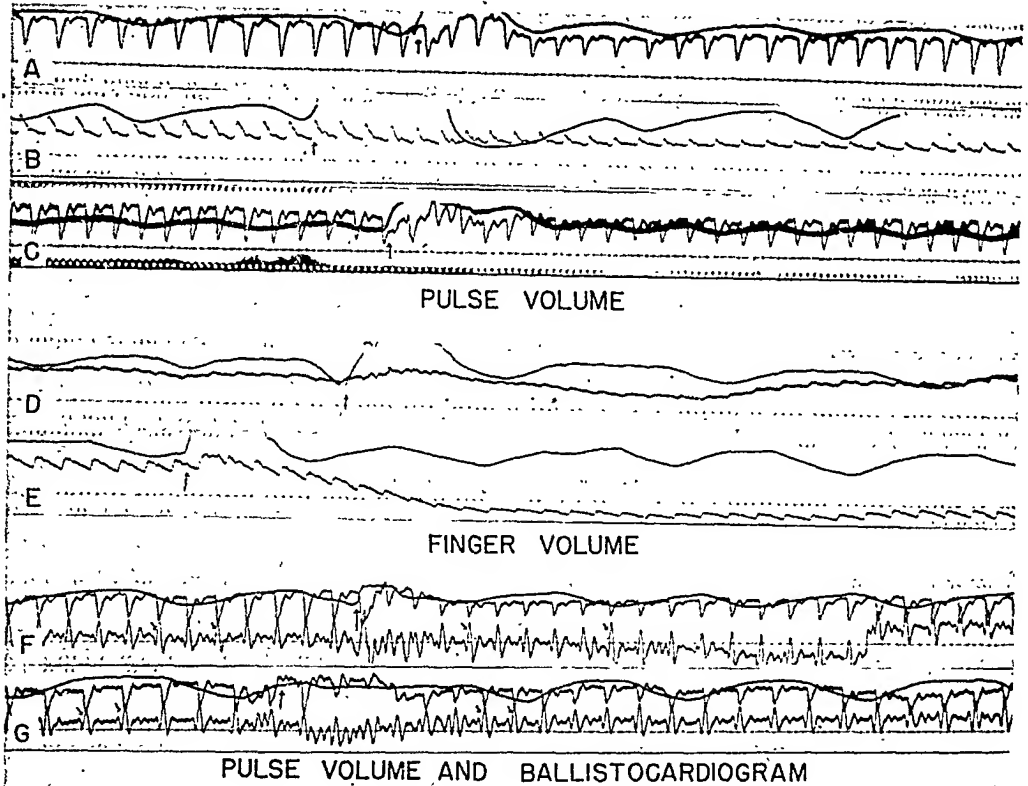


FIG. 1.

Seven records showing the effect of a forced expiration on pulse and finger volume. Respirations are recorded as the top undulating line, with expiration being an upward deflection. The time line is dotted: two-tenths of a second between large dots.

A, B, C are records of finger pulse volume taken with a piezo-electric crystal and do not show baseline changes (see text). Note the immediate decrease in amplitude of the pulse volume stroke in all cases immediately following a deep expiration (arrows). The recovery in B occurred in 16 seconds and is not shown. See Fig. 2 for graphs.

D and E were taken with a Frank capsule, and recorded total finger volume changes as well as individual pulse volumes. Note the fall in total finger volume and the decrease in pulse volume following a deep expiration (arrows). The recovery in E occurred within 30 seconds and is not shown.

F and G are records of finger pulse volume taken as in A with a simultaneous ballistocardiogram recorded below. Note that although the pulse volume diminishes directly after a deep expiration (large arrow), the IJ stroke of the ballistocardiogram (small arrows) does not. See Fig. 3 for graphs and text for interpretation.

volume (Fig. 1, 2). The average duration of effect was 16 seconds for diminished pulse volume and 24 seconds for the diminution of total finger volume. The individual response of pulse volume change can be determined from Fig. 2. The individual duration of total finger volume diminution varied from 10 to 40 seconds.

In the 4 cases where the ballistocardiogram was recorded with the pulse volume, the height of the IJ stroke of the ballistocardiogram in millimeters and the height of the

pulse volume stroke in millimeters was averaged for the control period in each case. The deviation from the respective control average of the height of each IJ stroke and pulse volume stroke in millimeters was plotted against the corresponding beat. These correlation graphs are shown in Fig. 3 and representative tracings are given in Fig. 1F and G. In no case was the total stroke volume decreased during or after the deep expiration, whereas the pulse volume had immediately decreased, and remained so even

EFFECT OF FORCED EXPIRATION ON PULSE VOLUME

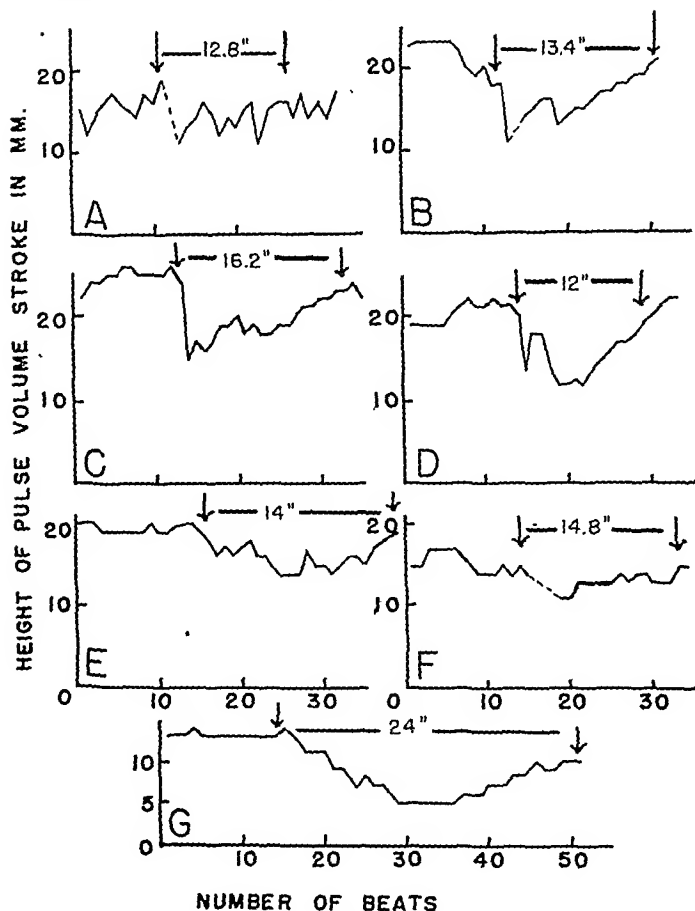


FIG. 2.

Graphs of the effect of a deep expiration on finger pulse volume in 7 cases. The height of the pulse volume stroke (recorded as in Fig. 1A) measured in millimeters is plotted against the corresponding beat. Note the immediate drop in pulse volume in all cases following a deep expiration (first arrow). The recovery time in seconds is indicated between the first and second arrows, in each case. The dotted lines in A, B, and F represent strokes which were not measurable because of artifacts caused by the motion of the subject.

though the corresponding ballistocardiogram beat was not smaller than a comparable control beat.

Discussion. A deep, forced expiration performed in the manner described produced a decrease in both total finger volume and pulse volume, in the 6 subjects studied. This confirms the reports of Uhlenbruck and de Lalla.

By using the height of the IJ stroke as a

measure of relative stroke volume^{5,6} the simultaneous ballistocardiogram may be interpreted as confirmatory evidence for reflex vasoconstriction since the only other probable cause for the observed diminution of pulse and total finger volume (*i.e.* diminished left ven-

⁵ Starr, I., Rawson, A., Schroeder, H., Joseph, N., *Am. J. Physiol.*, 1939, **127**, 1.

⁶ Brown, H. R., Jr., and de Lalla, V., Jr., Unpublished data.

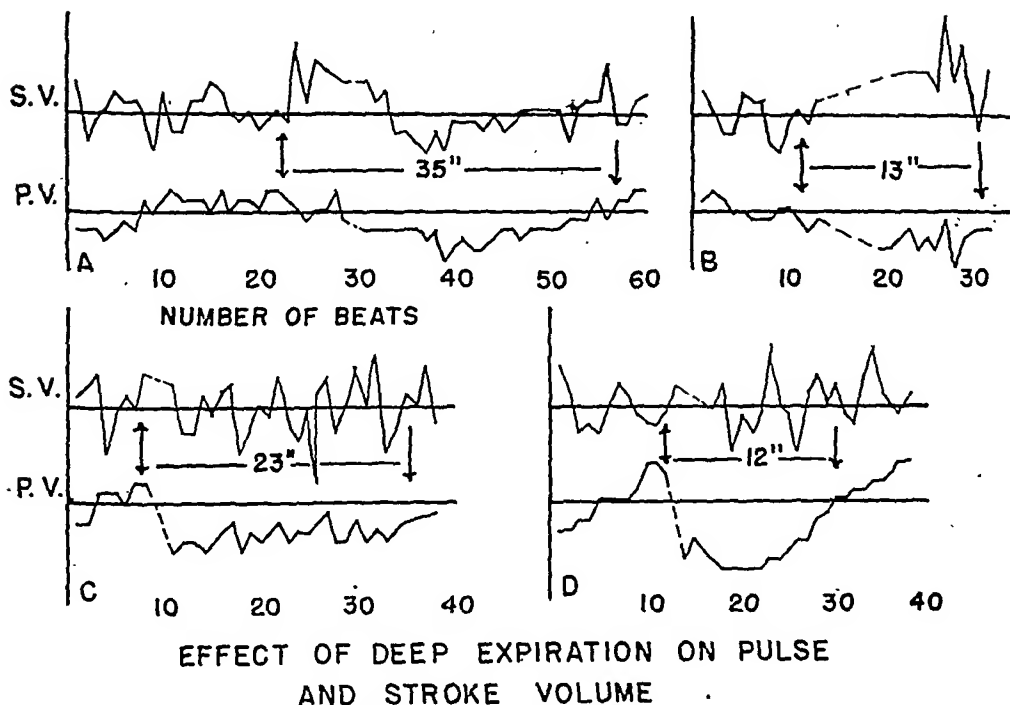


FIG. 3.

Four graphs of the effect of a deep expiration on the height of the finger pulse volume stroke and on the height of the IJ stroke of the ballistocardiogram (relative total stroke volume). The deviation in millimeters from the control average height of pulse volume and IJ stroke in millimeters is plotted against the corresponding beat in each case. Note that although the pulse volume curve (P.V.) drops after a deep expiration (first arrow), the relative stroke volume curve (S.V.) does not change significantly. The pulse volume recovery time is indicated in seconds between the first and second arrows in each case. The large swings of the S.V. curve reflect the respiratory variation of the total stroke volume. See Fig. 1F and G for representative tracings. The dotted lines represent strokes which were not measurable because of artifacts caused by the motion of the subject.

tricular output) does not seem to have occurred. (See Fig. 1F, G and 3). Since the IJ stroke reflects total stroke volume, and since the right ventricular stroke volume is known to decrease in expiration^{6,8}, then one might expect to find smaller IJ strokes during or after the forced expiration than in comparable control beats, if the left ventricular stroke volume had diminished or remained the same. That this was not observed indicates that the left heart stroke volume was increased during the forced expiration at the same time that the pulse volume was observed to have de-

creased. This deduction is sound in view of the report of Shuler *et al.*, which states that the left ventricular stroke volume increases with expiration.⁷ These findings suggest that a reflex vasoconstriction is the cause of the diminution of pulse and total finger volume incident to a forced expiration.

One of us had already postulated that stretch receptors in the large extrathoracic veins may be the origin of this reflex.⁴ However, it is not necessary that these receptors be located in the large veins themselves in order to respond to increased abdominal venous pressure. Gammon and Bronk⁹ have demonstrated Pacinian corpuscles in the

⁷ Shuler, R., Ensor, C., Gunning, R., and Moss, W., *Am. J. Physiol.*, 1942, **137**, 602.

⁸ Lauson, H. D., Bloomfield, R. A., and Cournaud, A., *Am. J. Med.*, 1946, **1**, 315.

⁹ Gammon, G. D., and Bronk, D. W., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 788.

mesentery which respond to engorgement of the neighboring tissue by raising the systemic blood pressure (*i.e.* arterial vasoconstriction). They also showed that these receptors would respond in the same manner to increased pressure in the abdominal vena cava. It is possible that these receptors are the initiators of the observed reflex.

Should this be true, then it is understandable why the expiration may not produce diminution of pulse and finger volume if not forced. It is likely that a critical intrathoracic pressure must be reached before the abdominal venous pressure is raised enough to stimulate the receptors. In that event, a deep expiration that does not do this will not elicit the reflex.

Summary. 1. Twelve records of the effect of a deep, forced expiration on finger pulse volume were obtained from six subjects. In four of these records a ballisto-

cardiogram was recorded simultaneously. In 5 other tracings total finger volume changes were recorded.

2. In all cases the pulse volume and total finger volume (when recorded) decreased immediately following the deep forced expiration. Average duration of diminution was 16 seconds for pulse volume and 24 seconds for total finger volume.

3. The absence of a simultaneous decrease in corresponding ballistocardiogram beats suggests that the observed finger changes were due to reflex arteriolar constriction, possibly initiated by abdominal pressoreceptors in the great veins or mesentery.

Appreciation is expressed to Prof. William S. McCann for his advice and criticism in the review of the manuscript, and to Prof. W. O. Fenn for his helpful suggestions during the course of the work.

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Location of Communications between Cognate Bed of Descending Ramus of Left Coronary and Adjacent Collateral Vascular Beds.*

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More than 40 years ago, Hirsch and Spalteholz¹ noticed that after ligation of a coronary artery, the area of infarction was much smaller than the myocardium apparently supplied by that artery (cognate bed). Two years later Miller and Mathews² made the same observations, and suggested that this was due to the presence of fine epicardial arterial anastomoses. Since that time, numerous additional reports have appeared describing the variability in the occurrence of and in the size of infarcts produced by coronary ar-

tery ligation.³⁻⁸ Sutton and Davis⁹ suggested tying the superficial anastomotic vessels in order to get a large infarct.

No actual studies, however, appear to have been done in order to demonstrate whether or not the blood supplied by the superficial

³ Smith, F. M., *Arch. Int. Med.*, 1918, **22**, 8.

⁴ Mendlowitz, M., Schauer, G., and Gross, L., *Am. Heart J.*, 1937, **13**, 663.

⁵ Karsner, H. T., and Dwyer, J. F., *J. Med. Research*, 1916, **34**, 21.

⁶ Smith, F. M., *Am. J. Med. Sc.*, 1918, **156**, 706.

⁷ Gold, H., Travell, J., and Modell, W., *Am. Heart J.*, 1937, **14**, 284.

⁸ O'Neill, J. F., and Thomas, W. C., *Personal communications*, 1947.

⁹ Sutton, D. C., and Davis, M. D., *Arch. Int. Med.*, 1931, **48**, 1118.

* Supported by a grant from the Life Insurance Medical Research Fund.

¹ Hirsch, C., and Spalteholz, W., quoted by L. Gross, 1921, Hoeber, N. Y.

² Miller, J. L., and Mathews, S. A., *Arch. Int. Med.*, 1909, **3**, 476.

vessels is the cause of the variability in the resulting infarctions. It was reasoned that if the principal collateral supply is through the superficial vessels, then if an artery were ligated and all superficial anastomotic branches were interrupted, an infarct of fairly predictable size should result. On the other hand, if a consistent infarct was not obtained, then the collateral blood supply must come by way of deep communicating vessels. This was tested by the following procedure:

Method. Mongrel dogs of both sexes and weighing 5 to 16 kg were anesthetized with intravenous sodium pentobarbital† (40 mg/kg), artificial respiration was administered and aseptic thoracotomies performed by subperiosteal rib resection.

Immediately before operation the dogs were given 10,000 units of penicillin. Postoperatively they received a total of 90,000 units in divided doses 3 times a day for 3 days. In the last 24 thoracotomies penicillin in emulsified oil (Pendil‡) was used to maintain the penicillin level through the 2 first postoperative nights with water soluble penicillin being given in the day. This materially reduced the number of injections required and was found to be much more convenient. In these series totalling 52 thoracotomies treated with penicillin there was not a single death from infection nor did any show more infection than a drop of pus around an unremoved skin suture. This is in marked contrast to Miller and Mathews¹² results of a death rate of 60% due to sepsis.

Three groups of procedures were carried out upon the hearts.

Group A. In 23 dogs the anterior descending arteries and the accompanying veins were ligated 1 to 2 cm below the bifurcation of the left coronary arteries. This is the region where there is usually a relatively large branch going to the left ventricular myocardium. In 11 of these dogs the main vessel and this large branch were individually tied; in 9 dogs the ligature was above the

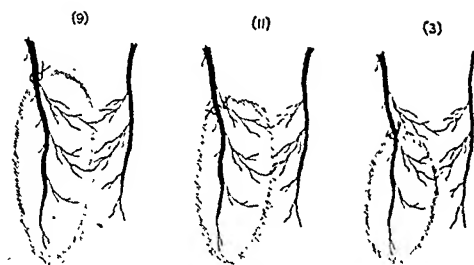


FIG. 1.

Diagram of the site of ligation of the descending ramus of the left coronary artery with the site of the superficial burn indicated by crosshatching. The figures at the top of the diagram indicate the number of animals receiving each type of ligation plus cautery. All of the above types are included in Group A.

first large branch; in 3 dogs the ligature was below the first main branch which was left intact (see diagram). The areas of distribution of the arteries were exposed and the edges touched with a hot iron cautery producing burns light brown in color and about 3-5 mm in width. Similar burns on excised and living hearts showed that burns of this type extend into the myocardium less than a millimeter. The chests were closed in layers (Fig. 1).

Group B. In 8 animals a ligation was done as described for the first 20 of Group A but cauterization was omitted.

Group C. In 4 animals cauterization as described was done without ligation.

All animals were sacrificed 8 to 14 days after operation.

Results. Table I. Six animals died within 24 hours after the ligation-cauterization procedure. The cause of death was probably ventricular fibrillation since no other cause could be found at autopsy. These animals were dropped from the series because their early deaths did not allow enough time for the development of gross evidence of infarction. Recently preoperative and postoperative intravenous procaine, 10 mg/kg at each administration, was added to the procedure. Of 6 dogs so treated, one died of fibrillation while the chest was still open. The remainder survived until sacrificed. The latter death casts some doubt on the efficacy of procaine in preventing the fibrillation which occasionally accompanies this procedure.

† Supplied by Premo Pharmaceutical Labs, Inc., New York City, N. Y.

‡ Supplied by Endo Products, Inc., Richmond Hill, N. Y.

TABLE I.
Results in Animals Sacrificed.

Groups	Gross infarction	No gross infarction	No. in group
Group A	17	0	17
" B	6	2	8
" C	0	4	4
Total in Groups A and B	23	2	25

The difference in occurrence of infarction was significant below the 5% level. In this case:

$$X^2 = \frac{(ad - bc)^2 N}{(a + b)(c + d)(a + c)(b + d)} = 4.619^{10}$$

The results on the remaining animals were as follows: *Group A.* Cautery and Ligation. All 17 of the hearts of the animals surviving the first 24 hours showed definite gross infarctions involving the full thickness of the myocardium and extending to the burned areas.

Group B. Ligation. Two of these showed extensive infarctions approximating those seen in Group A; 4 showed infarctions smaller than area apparently supplied by the artery; 2 showed no gross evidence of infarction. All areas of necrosis stopped abruptly at the right edge of the anterior vessels, *i.e.*, the necrosis did not involve the right ventricular myocardium. These were different than the ligated cauterized infarctions where the necrosis extended to the cauterized area on the right ventricle. None of the infarctions in either series involved the interventricular septum.

Group C. The only lesions found were adhesions between the burned area and the pericardium. None showed gross infarctions.

Discussion. The X^2 value of 4.619¹⁰ comparing incidence of infarction in Groups A and B, shows that the probability of chance occurrence lies well below the 5% level. There-

fore, we consider this difference to be probably significant.

Lowe¹¹ and Lowe and Wartman¹² have shown how branches from the epicardial vessels go to their respective layers of the myocardium. It appears possible from our studies that these small arteries may be end arteries, in the strict sense, and have only capillary and small arteriolar anastomoses with the other arteries supplying the same layers. The present study suggests that the deep small anastomosing vessels cannot prevent necrosis of a very large portion of myocardium when its epicardial supplying arteries are interrupted.

Summary and conclusion. The frequent failure to obtain infarcts and the variability in the size of the infarcts which follow ligation of a coronary artery in the dog suggest the presence of prominent anastomotic communications with collateral vascular beds. To determine whether these communications were in deep or superficial vessels, the consistency of infarction with simple coronary ligation was compared with the infarction which resulted from coronary ligation plus occlusion of the superficial vessels by cauterization about the cognate bed of the ligated vessels. Since the latter procedure produced infarctions of predictable size in all of the 17 dogs so treated, which survived for 24 hours or longer, it is concluded that the principal anastomotic communications with collateral vessels lie in the epicardium.

It is suggested that ligation plus superficial cauterization may be of definite value in providing a fairly well standardized procedure for the experimental study of myocardial infarction.

¹⁰ Chambers, E. G., Cambridge Univ. Press, 1940, Cambridge, England.

¹¹ Lowe, T. E., *Am. Heart J.*, 1941, **21**, 326.

¹² Lowe, T. E., and Wartman, W. B., *Brit. Heart J.*, 1944, **6**, 115.

Effect of Hypophyseal Growth Hormone upon the Urinary Nitrogen Excretion of Fasting Rats.*

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From The Institute of Experimental Biology and the Division of Physiology, University of California, Berkeley.

It was shown by Harrison and Long¹ that the administration of a saline extract of the anterior pituitary would decrease the urinary nitrogen excretion of fasting rats. It seemed of interest to determine if pure growth hormone would produce the same results.

Methods. The hormone used was prepared according to the previously published method.² All animals used were "plateued" female rats of the Long-Evans strain which weighed between 150 and 200 g and were from 5-8 months of age. There were 2 groups of 5 animals each and during the control periods each animal was fed 12 g of the stock diet[†] daily, this amount being eaten completely. The animals were kept in individual metabolism cages; urine collections were made daily; and the urine was analyzed by the micro-Kjeldahl procedure for its total nitrogen content.

Following a 5-day preinjection control period, all animals were fasted for 2 days and the animals in Group 1 were given intraperitoneally 200 γ of growth hormone per day. (The dose was divided so that the hormone was given in 100 γ doses each morning and

evening.) The animals in Group 2 served as the uninjected controls. At the end of the 2-day fasting period, hormone administration was stopped and the animals were again fed 12 g of diet per day. At the end of a second control period of 10 days the rats were again fasted and hormone administration again started. During this injection period the animals in group 2 received the same dose of hormone as above, while the previously injected animals served as the controls. The fast was continued for 5 days, 2 of the control rats dying on the 5th day. Growth hormone injections were continued for 6 days after termination of the fast. Urinary nitrogen excretion was then determined for a subsequent 9-day post-injection control period.

Results and Comment. The data of the experiment are presented graphically in Fig. 1 in which the average daily nitrogen excretions of each group are plotted. It will be seen that the average nitrogen excretion of the growth hormone-treated animals was lower than that of the controls during each fasting period. The average nitrogen excretions of the injected animals during the first and second fasting periods were 91 and 64 mg per day respectively as compared to 116 and 126 mg per day excreted by the controls during the same periods. These were compared statistically according to the methods of Fisher³ and a *p* value of 0.02 and of less than 0.01 were obtained for the first and second fasting periods respectively.

It is also of interest to note that following the re-institution of feeding after the second fasting period the nitrogen excretion of the animals receiving growth hormone remained

* Aided by grants from the American Cancer Society (through the National Research Council, Committee on Growth), the National Institute of Health RG 409, and The Research Board, University of California.

¹ Harrison, H. C., and Long, C. N. H., *Endocrinology*, 1940, 26, 971.

² Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1945, 159, 353.

[†] The diet fed consisted of ground whole wheat 67.5%, casein 15.0%, whole milk powder 10.0%, NaCl 0.75%, CaCO₃ 1.5%, hydrogenated vegetable oil (Crisco or Primex) 5.25%. To each kg of diet were added 3.5 g of Sardilene (fish oil concentrate containing 3000 USP units of vitamin A, and 400 chick units vitamin D per g).

³ Fisher, R. A., *Statistical Methods for Research Workers*, 10th Ed., Edinburgh, London, Oliver and Boyd (1946).

Effect of Growth Hormone on the Nitrogen Excretion of Fasting Rats

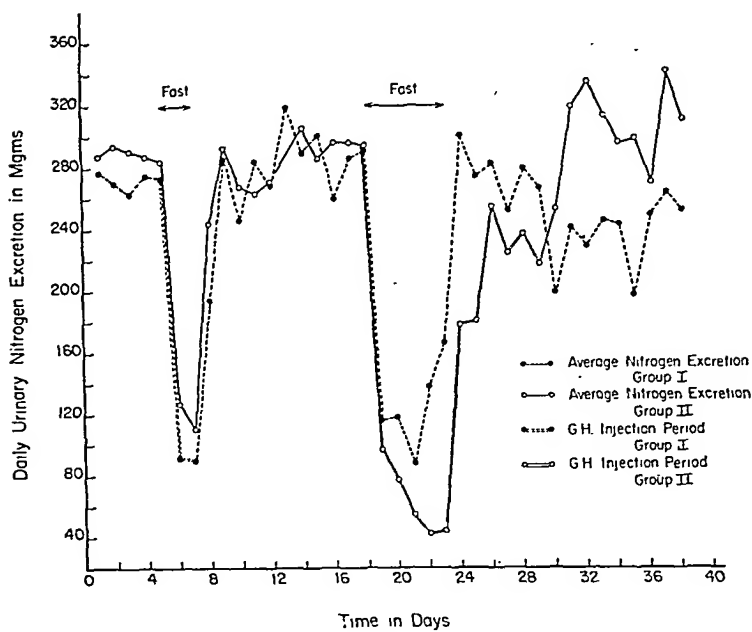


FIG. 1.

below that of the controls. Following the cessation of growth hormone administration there was an increase in nitrogen excretion comparable to that previously described.⁴ The 2 animals in the control group that died showed the typical pre-mortal rise in nitrogen excretion while none of the treated animals showed a similar rise during fasting.

That growth hormone has a protein anabolic effect in fed animals is well shown by

⁴ Gordan, G. S., Bennett, L. L., Li, C. H., and Evans, H. M., *Endocrinology*, 1948, **42**, 153.

the fact that it produces general body growth and nitrogen retention.⁴ That it reduces urinary nitrogen excretion in fasting rats is evidence that its *net* effect is to reduce protein catabolism under these conditions. However, this type of data does not permit any critical evaluation of the mechanism of action of growth hormone on protein metabolism.

Conclusion. Growth hormone at a dose level of 200 γ per day reduces the urinary nitrogen excretion of fasting adult female rats.

Relationship of the Pituitary to the X zone of the Mouse Adrenal.*

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The relationship of the X zone of the mouse adrenal¹⁻³ to the pituitary is not known. It was considered that the use of the technic of hypophysectomy might produce results of help in the elucidation of this problem.

Material and Methods. Virgin female "Swiss albino" mice, aged 40-42 days at the commencement of the experiment, were used. The adrenal X zone is well developed at this age. Hypophysectomy was performed by the parapharyngeal approach. Injection of the hormones noted below was started 24 hours after the operation (except that the chorionic gonadotropin injections were commenced 48 hours after operation) and the animals killed 24 hours after the last injection. The mice were grouped as follows:

1. Twelve intact untreated animals killed at 54-58 days of age.

2. Eleven animals hypophysectomized for 12 days and 8 for 14 days and then killed.

3. Nine hypophysectomized animals injected with a pituitary preparation, principally adrenocorticotropin† in nature, 1 mg per day divided into three intraperitoneal in-

jections, for 12 days.

4. Nine hypophysectomized animals injected subcutaneously with 1 mg of pituitary gonadotropin, principally LH† in nature, for 10 days.

5. Five hypophysectomized animals injected subcutaneously with 1 mg per day of pituitary gonadotropin, principally FSH† in nature, for 10 days.

6. Six hypophysectomized animals injected intraperitoneally with 11 I.U. of human chorionic gonadotropin ("Korotrin") per day for 10 days.

The right adrenal was treated routinely after fixation in Bouin's and stained in Harris' haematoxylin and eosin or Heidenhain's Azan. The left adrenals were fixed for histochemical studies and are not being reported upon here. The thymus, uterus and ovaries were weighed; the ovaries were then treated as for the right adrenal. The completeness of hypophysectomy was judged by the examination of serial sections of the appropriate area in 2 cases from each group; in the remainder the success of the operation was judged by the examination of the sella turcica region under binoculars (x 15). Added confirmation was given by the weight of the animal and, where relevant, by the weights of the uterus and ovaries and the histological condition of the ovaries and adrenals.

Results. The intact untreated animals showed a well-developed X zone with deep eosinophilia of the cytoplasm and large vesicular basophil nuclei. After 12 to 14 days of hypophysectomy the X zone became more compact,[§] the cytoplasm of the cells had

* Aided in part by a grant from the Commonwealth Fund.

† Fellow of the Commonwealth Fund, on leave of absence from the Department of Zoology, Liverpool University.

1 Howard-Miller, E., *Am. J. Anat.*, 1927, 40, 25.

2 Parkes, A. S., *Phys. Rev.*, 1945, 25, 203.

3 Jones, I. Chester, *Q.J.M.S.*, 1948, 89, 53.

§ The author is very grateful to the Armour Company for the ACTH and LH preparations and to the Schering Corporation for the FSH. The ACTH used was lot 42B about 36% potency of the Armour La-1-A standard; see Forsham, P. H., et al., *J. Clin. End.*, 1948, 8, 15, for discussion of this and of the type of contaminants. The LH type of gonadotropin was Lot FW-234, the main contaminants being posterior lobe hormones; there was slight FSH activity. The FSH type of gonadotropin ("Synergist") produced good follicle development in the ovaries of hypophysectomized mice with some luteinization.

§ Leblond, C. P., and Nelson, W. O., (*Bull. Hist. Appl.*, 1937, 14, 181), note the disappearance of the X zone after hypophysectomy but their data include only four mice which would have had an X zone at the time of operation and the length of time for which these were hypophysectomized is not made clear. The "brown degeneration" occurring in another series⁴ did not appear in the hypophysectomized animals in this experiment.

shrunk and stained only faintly with eosin; the nuclei were irregular and stained deeply with haematoxylin (remains of the X zone can be seen up to 100 days after hypophysectomy⁴). The zona fasciculata showed the atrophy characteristic of the adrenals of other animals after hypophysectomy;⁵ the zona glomerulosa showed no change in the routine histological preparations. In the ACTH injected animals the X zone was unaffected but the rest of the cortex resembled that of the normal animal. In the LH injected animals the X zone was maintained in a condition similar to that of the normal animal; the zona fasciculata was unaffected and showed the atrophy characteristically appearing after hypophysectomy; the zona glomerulosa was not affected. In the FSH injected animals the X zone had not degenerated as far as in the hypophysectomised controls but the degree of maintenance was much less than in the LH injected animals; the rest of the cortex had an appearance typical of that of the hypophysectomised animal. In the animals injected with chorionic gonadotropin the X zone had disappeared and a medullary connective tissue capsule had formed; the remainder of the cortex was unaffected by the hormone.

Discussion. The X zone depends upon the pituitary for the maintenance of its normal histological structure and a pituitary gonado-

tropin seems to be the maintaining factor. This gonadotropin is possibly LH, the effect of the FSH preparation then being due to the contaminating LH. The rich LH secretion of castrate animals^{6,7} would account for the persistence and growth of the X zone of the prepuberally castrated male mouse and for the evocation of a "secondary X zone" in the mouse castrated when adult.

The chorionic gonadotropin, which has some physiological properties in common with LH, swept the X zone away, paralleling its action in the intact animal.⁸ It is equivalent in its results to the direct action of testosterone on the X zone of the *hypophysectomised* prepuberally castrated male mouse.⁹ In normal animals, then, it may be that the maintaining factor of the pituitary is overridden, in the male at puberty by the direct action of androgens on the X zone and in the female early in first pregnancy by an "anterior pituitary-like" hormone arising from the conceptus or the endometrium.¹⁰ The X zone seems to be independent of ACTH which indeed may be concerned mainly with the zona fasciculata.⁵ The results require confirmation by the use of chemically pure pituitary factors when these are available.

⁶ Hellbaum, A. A., and Greep, R. O., *Am. J. Anat.*, 1940, **67**, 287.

⁷ Greep, R. O., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 214.

⁸ Takekawa, K., *J. Facult. Sci.*, Tokyo, 1935, **4**, 83.

⁹ Jones, I. Chester, 1948, in preparation.

¹⁰ Rowlands, I. W., *J. Endocrinology*, 1947, **5** (3), Supp. p. xx.

⁴ Jones, I. Chester, *Anat. Rec.*, 1948, **100**, (4) Supp., p. 49 and unpublished.

⁵ Deane, H. W., and Greep, R. O., *Am. J. Anat.*, 1946, **79**, 117.

Deleterious Effects of Pancreas in the Hyperthyroid Rat.*

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In preliminary experiments on the effects of feeding various tissues on symptoms of thyrotoxicosis in the rat, it was observed that immature rats fed diets containing both desiccated thyroid and pancreas lived but a fraction of the time of animals fed similar diets with either thyroid or pancreas omitted. The present experiment was undertaken to obtain further data on this interrelationship.

Procedure and Results. Four basal rations were employed in the present experiment: diets A, B, C, and D. Diet A was a synthetic ration. Diets B, C, and D were similar in composition but contained 10% pancreas, activated pancreas† or papain, added in place of an equal amount of sucrose. All 4 rations were supplemented with 0.0 and 5.0 g of U.S.P. desiccated thyroid per kg of diet. Sixty-four female rats of the Long-Evans strain were selected at 21 to 23 days of age and an average weight of 45.4 g for the present experiment. Animals were kept in metal cages with raised screen bottoms to prevent access to feces, and were fed *ad lib.* the diets listed in Table I. Feeding was continued for 28 days or until death, whichever occurred sooner.

* This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 216 in the series of papers approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army. Contribution No. 193 from the Department of Biochemistry and Nutrition, University of Southern California.

† Activated pancreas is desiccated pancreas whose trypsinogen has been converted into trypsin. We are indebted to Mr. Ezra Levin of the VioBin Corporation, Monticello, Ill., for these preparations.

Results are summarized in Table II. Findings indicate that immature female rats fed diets containing both pancreas and desiccated thyroid lived but a fraction of the time of animals fed similar diets with either pancreas or thyroid omitted. On diet A₁ 100% of the rats survived the experimental period of 28 days. On diets B₁ and C₁ (containing pancreas and activated pancreas respectively) the mortality rate was 100% with an average survival time of 15.5 and 7.4 days respectively. Findings with papain (diet D₁) were substantially the same as those with pancreas preparations. A marked retardation in growth was observed in animals fed diets B₁, C₁ and D₁. In the activated pancreas series (diet C₁) 9 of the 10 rats in this group lost weight during the first week of feeding. On diets B₁ and D₁ the average gain in weight during the first week of the experiment was less than 10 g in contrast to an average gain of 24.7 g on diet A₁ and approximately 20 g for rats fed control rations (diet A₂, B₂, C₂ and D₂). No significant difference in growth was observed on the 4 diets employed in the absence of dietary thyroid, although gain in body weight was somewhat greater on the synthetic ration (diet A₂) than on diets containing pancreas or papain. All rats fed thyroid-free rations survived the experimental period of 28 days.

No data are available concerning the cause of death in animals fed diets containing desiccated thyroid and either pancreas or papain. Since both of the latter substances contain proteolytic enzymes and since activated pancreas (in which the trypsinogen is converted into trypsin) was more active than the ordinary desiccated material, it was felt that the deleterious effects of pancreas or papain in the thyroid-fed rat may have been due to

TABLE I.
Composition of Experimental Diets.

Dietary component	Diet A ₁ and A ₂	Diet B ₁ and B ₂	Diet C ₁ and C ₂	Diet D ₁ and D ₂
Pancreas*	0	10	0	0
Activated pancreas†	0	0	10	0
Papain‡	0	0	0	10
Casein§	22	22	22	22
Salt mixture	4.5	4.5	4.5	4.5
Sucrose	73.5	63.5	63.5	63.5

To each kg of the above diets were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-naphthoquinone 10 mg, and choline chloride 1.2 g. Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol acetate 1.5 mg, and a vitamin A-D concentrate¶ containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

U.S.P. desiccated thyroid (Armour) was incorporated in diets A₁, B₁, C₁, and D₁ at a level of 5.0 g per kg of diet, replacing an equal amount of sucrose.

* Desiccated Pancreas, VioBin Corporation, Monticello, Ill.

† Activated Pancreas, VioBin Corporation, Monticello, Ill.

‡ Frenco's Papain Absolute—Powder, Frenco Laboratories, Nogales, Ariz.

§ Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

|| Sure's Salt Mixture No. 1.

¶ Nopco Fish Oil Concentrate assaying 800,000 U.S.P. units of vit. A and 80,000 U.S.P. units of vit. D per g.

TABLE II.
Effects of Pancreas Feeding on the Survival Time of Hyperthyroid Rats.

Dietary group	No. of animals	Initial wt (g)	Gain in body wt 28-day period* (g)	% surviving†	Avg survival time of decedents* (days)
Desiccated thyroid series.					
A ₁	10	45.1	65.9 ± 4.6 (10)	100	—
B ₁	10	45.2	—	0	15.5 ± 1.7
C ₁	10	45.4	—	0	7.4 ± 0.6
D ₁	10	45.2	25.0 ± 4.3 (2)	20	18.6 ± 1.2
Control series.					
A ₂	6	45.5	91.8 ± 4.5 (6)	100	—
B ₂	6	45.2	77.1 ± 3.1 (6)	100	—
C ₂	6	45.0	76.3 ± 2.8 (6)	100	—
D ₂	6	45.5	73.7 ± 4.7 (6)	100	—
Thyroxin series.					
A ₂ ‡	6	45.5	82.8 ± 1.9 (6)	100	—
C ₂ ‡	10	45.7	—	0	11.6 ± 0.6

The values in parentheses indicate the number of animals which survived and on which averages are based.

* Including standard error of the mean calculated as follows: $\sqrt{\frac{\sum d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

† Experimental period, 28 days.

‡ Each rat in this series received intraperitoneal injections of 200 γ thyroxin 3 times weekly during the course of the experiment.

greater digestion and absorption of the thyroid material. Such, however, does not appear to be the case for subsequent findings indicate the deleterious effects of pancreas feeding may be demonstrated following parenteral administration of thyroxin. The latter experiment was conducted with 16 female rats of

the Long-Evans strain similar in age and weight to those employed in the earlier studies. Six of these rats were fed the synthetic ration (died A₂); the remaining 10 were fed a diet containing the activated pancreas preparation (diet C₂). Three times weekly each rat received intraperitoneal in-

jections of 200 γ thyroxin.[†] Injections were continued for 28 days or until death, whichever occurred sooner. All animals fed the synthetic ration (diet A₂) and receiving the thyroxin injections survived the experimental period of 28 days. All animals fed activated pancreas (diet C₂) and administered thyroxin died (average survival time 11.6 days). These findings indicate that the dele-

terious effects of pancreas feeding in the thyroid-fed rat were not due to increased digestion or absorption of thyroactive material.

Summary. Immature female rats failed to survive when fed purified rations containing both pancreas and desiccated thyroid. Length of survival was significantly prolonged if either pancreas or thyroid were eliminated from the experimental ration. The deleterious effects of pancreas in the thyroid-fed rat were not due to increased digestion or absorption of thyroactive material.

[†] Thyroxin (Synthetic Cryst.), Roche-Organon, Inc., Nutley, N. J. The material was dissolved in .1 N NaOH, adjusted to a pH of 8.0 and diluted to a volume containing 200 γ thyroxin per cc.

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Production of Hemagglutinin by Mumps and Influenza A Viruses in Suspended Cell Tissue Cultures.*

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The property of agglutinating red blood cells characteristic of certain viruses such as mumps and influenza has provided a simple method of estimating their concentration in infected materials. The procedure however, insofar as we are aware, has not been applied to viruses propagated in tissue cultures. Accordingly, experiments were undertaken with the purpose of ascertaining (a) whether the mumps virus would proliferate in suspended cell tissue cultures of the Maitland type and (b) whether this virus and influenza A virus would in such media produce detectable levels of hemagglutinin.

Methods. Preparation and maintenance of cultures. Cultures were prepared in a manner similar to that described by the Maitlands.¹ The nutrient medium consisted routinely of 3 parts of a balanced salt solution made up according to the formula of

Hanks[†] and 1 part of Simm's ox-blood serum ultrafiltrate.[‡] This mixture will hereafter be referred to as "HS." In the experiments with mumps virus, the tissue employed consisted of amniotic membrane from 8- or 9-day-old chick embryos. The membranes were suspended in a small amount of "HS", minced with scissors to obtain fragments approximately 1 mm in diameter, and centrifuged for 2 minutes at 800 r.p.m. in a graduated centrifuge tube. The fragments were then resuspended in a total volume of "HS" six times that of the sedimented tissue. Using a large bore Pasteur pipette, 3 drops of this

[†] The balanced salt solution as recommended by Dr. J. H. Hanks, contained the following ingredients expressed as g/l: NaCl, 8.0; KCl, 0.4; MgSO₄ · 7 H₂O, 0.1; MgCl₂ · 6 H₂O, 0.1; CaCl₂, 0.14; Na₂HPO₄, 0.06; KH₂PO₄, 0.06; glucose, 1.0. To each liter, 5 cc 0.4% phenol red was added. After autoclaving at 10 lb. for 10 minutes, 0.5 cc 1.4% isotonic NaHCO₃ was added per 20 cc of the above.

[‡] Purchased from Microbiological Associates Laboratories, Flemington, N. J.

* Aided in part by a grant from the National Foundation for Infantile Paralysis, Inc.

¹ Maitland, H. B., and Maitland, M. C., *Lancet*, 1928, 2, 596.

tissue suspension were transferred to 25 cc Erlenmeyer flasks containing 3 cc of "HS" and fitted with rubber stoppers. In the experiments with influenza virus, the cultures were similarly prepared employing amniotic membrane, allantoic membrane, or embryonic brain tissue obtained from 8-day-old eggs. All cultures were incubated at 35°C.

Their subsequent handling differed from that usually employed with suspended cell cultures in that the nutrient fluid was replaced at intervals as indicated below. This was accomplished by tilting the flasks, allowing the tissue to settle, and removing as completely as possible the supernatant fluid from each flask with a fine bore Pasteur pipette; 3 cc of fresh "HS" were then added. In certain of the experiments subcultures were carried out by transferring aliquots of the pooled supernatant fluids thus removed to flasks containing freshly prepared tissue fragments.

Determination of hemagglutinin and egg infectivity titers. Using a modified Salk technic² the hemagglutinin titers were determined of the stock viruses and of the individual supernatant fluids removed periodically from the tissue cultures. The tests on the supernatant fluids were performed as soon as they were removed, using two-fold dilutions and beginning with a final dilution of 1:2. Pooled erythrocytes obtained from the same 4 hens were employed throughout. The highest final dilution of the supernatant fluid giving complete agglutination was taken as the endpoint. The results were expressed as a geometric mean of the individual hemagglutinin titers of the supernatant fluids from each set of cultures.

In the experiments on the growth of mumps virus in tissue cultures, the infectivity for chick embryos of the original inoculum and certain of the supernatant fluids removed at various times was determined. Aliquots of the supernatant fluids were pooled and centrifuged for 15 minutes at 1000 r.p.m. and the sediment discarded. Serial ten-fold dilutions

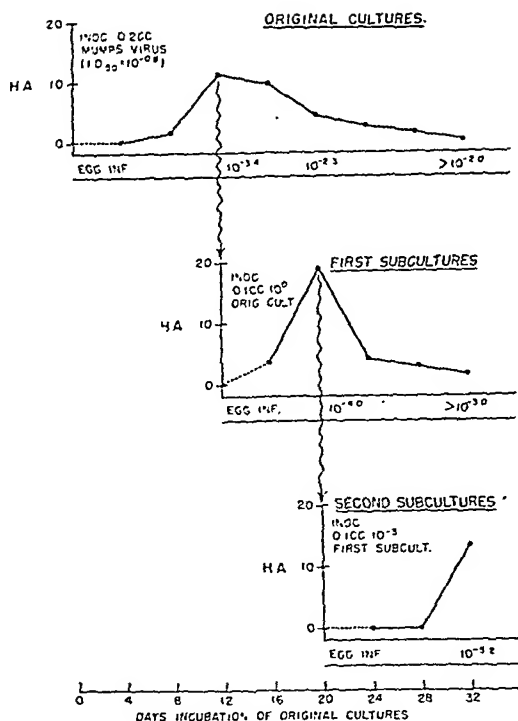


FIG. 1.

Mean hemagglutinin and infectivity titers of tissue cultures inoculated with mumps virus.

The hemagglutinin titer (HA) is expressed as the reciprocal of the geometric mean of the individual titers of the supernatant fluids from 4 cultures. The infectivity titers are expressed as the dilution of the pooled supernatant fluids from 4 flasks representing the ID₅₀ dose for embryonated hen's eggs calculated according to the method of Reed and Muench.³

were then prepared in isotonic phosphate buffer (pH 7.1) and 7-day-old eggs in groups of 5 or 6 were inoculated intraamniotically with 0.1 cc of each dilution. Six or 7 days later, the amniotic fluids were harvested and tested individually in a final dilution of 1:8 for the presence of hemagglutinins. The presence of complete hemagglutination in this dilution was taken as evidence of infection. From the data so obtained the 50% infectivity endpoint was calculated by the method of Reed and Muench.³ No assay of the infectivity of the cultures inoculated with influenza virus was made.

Experimental. (1). *Cultivation of mumps*

³ Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, 27, 493.

² Enders, J. F., and Levens, J. H., In: *Diagnostic Procedures for Virus and Rickettsial Diseases*. American Public Health Association, New York, 1948, pp. 139-164.

virus. A typical experiment which is summarized in Fig. 1 will be described. The mumps virus employed as the initial inoculum consisted of pooled amniotic fluid derived from the 42nd egg passage of a strain originally isolated and maintained in this laboratory (Enders strain). This material had a hemagglutinin titer of 512 and an ID_{50} of $10^{-3.8}$ in eggs. Six tissue cultures were prepared with minced amniotic membrane. Four flasks were each immediately inoculated with 0.2 cc of a 10^{-3} dilution in isotonic phosphate buffer of the virus. The two uninoculated cultures were kept as controls. At 4-day intervals the supernatant fluids were removed and 3 cc of fresh "HS" were then added. In this way the original cultures were maintained over a 32-day period. The hemagglutinin titers of all the individual supernatant fluids were determined and the egg infectivity titers of the pooled supernatant fluids removed on the 12th, 20th and 32nd day after inoculation were established.

During this period of 32 days two subcultures were carried out. After the original cultures had been incubated 12 days, with two changes of "HS" in the interim, equal volumes of the supernatant fluids removed on the 12th day were pooled, centrifuged for 15 minutes at 1000 r.p.m. and the sediment discarded; 0.1 cc of the undiluted pooled supernatant fluid was then added to each of 4 newly prepared flasks containing minced amniotic membrane and "HS". The supernatant fluids from the control flasks were handled in an identical manner and 2 new control cultures set up. Flasks of the first subculture group were maintained 20 days with changes of the supernatant fluids at 4-day intervals.

The second subcultures were inoculated with material removed from the first subcultures on the eighth day of incubation. The supernatant fluids from the latter were pooled and centrifuged, diluted 10^{-3} in isotonic phosphate buffer and added in a volume of 0.1 cc to each flask containing newly prepared tissue. The second subculture flasks were incubated for 12 days, with change of the supernatant fluids every 4 days.

(2) Cultivation of Influenza A virus. Cul-

tures prepared with minced amniotic membrane, chorioallantoic membrane or brain were inoculated with 0.1 cc of infected allantoic fluid containing the PR8 strain of Influenza A virus diluted 10^{-2} in isotonic phosphate buffer. In one experiment, flasks of each type of tissue were inoculated immediately after they were prepared, while to others the virus was added only after an interval of 6 days. In another experiment the virus was introduced 4 days after the cultures had been prepared and the supernatant fluid changed once. At various intervals, usually each day, 0.5 cc of the supernatant fluid was removed and replaced with the same volume of fresh "HS." The concentration of hemagglutinin in each specimen of fluid was determined.

Results. 1. *Mumps virus*. The results of the hemagglutinin and infectivity titrations carried out on the supernatant fluids removed from the tissue cultures are summarized in Fig. 1.

From these data it is evident that the mumps virus rapidly increases in cultures of this type. The original cultures were inoculated with 0.2 cc of diluted stock virus having a calculated ID_{50} titer for egg embryos of $10^{-0.8}$; thirty-two days later the ID_{50} titer of the pooled supernatant from the second subculture was $10^{-5.2}$. Not taking into account the quantity of virus discarded in the supernatants and assuming that each routine addition of nutrient fluid resulted in a 1:15 dilution of the virus, it may be calculated from the data included in Fig. 1 that an increase of the order of 1.3×10^{17} occurred in the egg infectivity titer. Likewise, during the experimental period an increase in the hemagglutinin titer of the order of 3.3×10^{14} occurred.

Active tissue growth of apparently the same degree was observed in all flasks irrespective of whether or not the virus had been inoculated. About the 6th to 8th day of cultivation free-floating, thin-walled cyst-like structures became numerous in the cultures of amniotic membrane. The cyst-like forms were no longer apparent after the 20th day. Between the 12th and 16th day, fixation of some of the tissue fragments to the bottom of

the flask was noted. These could be seen under low magnification to be surrounded by a zone of proliferating cells. In the cultures maintained 32 days there was an apparent decrease in the rate of growth after the 28th day; at this time many of the fragments previously adherent to the glass became loose and floated in the fluid.

Morphologic evidence of growth was accompanied by a fall in pH. Thus, during the 4-day interval between the changes of nutrient fluid, the pH, as estimated colorimetrically, fell from 7.6-7.7 to 6.9-7.1. This shift occurred throughout each 4-day period until the 28th day of cultivation in the original series. Thereafter, coincident with the apparent decrease in the rate of growth, the pH did not go below 7.3-7.5.

2. *Influenza virus*. The results with 3 different series of cultures have shown that the PR8 strain of Influenza A virus multiplies rapidly in the type of tissue culture employed as indicated by the early appearance and subsequent increase of hemagglutinin in the supernatant fluid. Thus in one experiment in which freshly prepared cultures of allantoic membrane, amniotic membrane and brain were employed, the geometric mean hemagglutinin titers after 72 hours of 2 cultures of each kind of tissue were 91, 6, and 32 respectively. Since the hemagglutinin titer of the virus inoculum was 512 and the dilution factor 3000, it can be calculated that the concentration of hemagglutinin during the period of cultivation had increased 535 times in the cultures of allantoic membrane, and 188 X and 35 X respectively in those of brain and amniotic membrane. The comparatively low titer obtained with the cultures of amniotic membrane should not be regarded as evidence for the failure of the virus to multiply as actively in this tissue, since in other experiments the amount of hemagglutinin produced was equivalent to that encountered in cultures of allantoic membrane and brain. In this instance the fact that relatively small amounts of amniotic tissue were employed probably was responsible for the low yield of hemagglutinin. It is of interest to observe that the embryonic brain tissue supported

the multiplication of the virus almost as well as the allantoic membrane. Whereas neurotropic strains of influenza virus have been developed by Stuart-Harris and others⁴⁻⁷ it is generally held that the PR8 strain has no affinity for nervous tissue.

Similar results were obtained in respect to the increase in hemagglutinin in tissue cultures which had been maintained for 4 or 6 days before the virus was inoculated. On the whole, however, the titers were somewhat lower. For example, 72 hours after inoculation of the virus, the geometric mean titers of two cultures of each of the 3 kinds of tissue were 8, 11, and 6 for allantoic membrane, amniotic membrane and brain respectively. These cultures had been prepared 6 days before the virus was added. Similarly in cultures of allantoic membrane prepared 4 days before inoculation the mean hemagglutinin titer did not exceed 16 after 72 hours.

Determinations of hemagglutinin titers at 12, 16, 20, 24, 48 and 72 hours and at certain intervals thereafter showed that the maximum production was attained between the 2nd and 3rd day. The hemagglutinin was present, however, in low concentration as early as 16 hours. By removing on the 4th day following inoculation all the supernatant fluid and replenishing with fresh material, it was found that thereafter a slow increase in hemagglutinin may gradually occur. But the titer attained did not exceed 2 even after an interval of 10 days and this was observed only in a series of cultures that were inoculated with the virus immediately after their preparation.

Discussion. It has been demonstrated that an egg-adapted strain of mumps virus multiplies actively in tissue cultures consisting of fragments of amniotic membrane of the chick embryo suspended in a mixture of ox serum ultrafiltrate and balanced salt solution. In

⁴ Stuart-Harris, C. H., *Lancet*, 1939, 1, 497.

⁵ Francis, T., Jr., and Moore, A. E., *J. Exp. Med.*, 1940, 72, 717.

⁶ Henle, G., and Henle, W., *Science*, 1944, 100, 410.

⁷ Vilches, A., and Hirst, G. K., *J. Immun.*, 1947, 57, 125.

this medium its multiplication is sufficient to produce measurable quantities of hemagglutinin in the supernatant fluid even when small inocula are employed. It is therefore possible within certain limits to estimate with ease the capacity of the virus to multiply under these conditions. It has also been determined that Influenza A virus (PR8 strain) increases in this type of medium to a level at which its hemagglutinating property can also be readily assayed.

The development of measurable amounts of hemagglutinin in tissue cultures is of significance, since it provides a swift and convenient means of following the rate and degree of multiplication of the mumps and influenza viruses in a system which, compared with a medium such as the chick embryo or the mouse, is far less complex. The technic, therefore, might be applied profitably to the study of many problems, for example, the

effect of various chemical compounds in accelerating or inhibiting the growth of these viruses.

It is noteworthy that the rate of multiplication of influenza A virus was found to be much more rapid than that of mumps. This difference has been previously observed in experiments with infected chick embryos and would seem, as Ginsberg, Goebel and Horsfall⁸ have indicated, to reflect a basic difference in the biologic properties of the two agents.

Conclusions. 1. An egg-adapted strain of mumps virus has been cultivated in suspended cell tissue cultures. 2. In such media the mumps virus as well as Influenza A virus (PR8 strain) produce measurable quantities of hemagglutinin.

⁸ Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., *J. Exp. Med.*, 1948, **87**, 385.

16639

Magnesium Deficiency in the Sexually Mature Rat.*

G. P. BARRON, P. B. PEARSON, AND S. O. BROWN.

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That the young rat requires an adequate dietary intake of magnesium for normal life processes was first established by Kruse, Orent, and McCollum.¹ As shown by Tufts and Greenberg, two phases seem to be observed in magnesium deficiency in young rats.² The first is marked by vasodilatation, hyperemia, and hyperexcitability. The second phase is characterized by nutritive failure, cachexia, and kidney damage. There have been controversial reports as to the pathological involvement of the skin in magnesium deficiency in rats. Kruse and his co-workers reported definite trophic changes in the skin and nails of young animals on a diet deficient in magnesium. Likewise, other workers reported skin lesions in animals deprived of magnesium.^{3,4} On the contrary, Tufts and Greenberg and others did not observe dis-

turbances in the skin of young animals raised on diets deficient in magnesium.^{5,6} Greenberg suggested that the trophic conditions observed by several workers were due to the experimental animals not receiving various constituents of the vitamin B complex in sufficient quantities.⁷

* Aided by a grant from the Dow Chemical Company, Freeport, Texas, through the Texas A & M Research Foundation.

¹ Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, 1932, **96**, 519.

² Tufts, E. V., and Greenberg, D. M., *J. Biol. Chem.*, 1938, **122**, 693.

³ Wateborn, E., and McCance, R. A., *Biochem. J.*, 1937, **31**, 1379.

⁴ Shrader, G. A., Prickett, C. O., and Salmon, W. D., *J. Nutrition*, 1937, **14**, 85.

⁵ Brookfield, R. W., *Brit. Med. J.*, 1934, **1**, 848.

TABLE I.
Effect of Magnesium on the Weights of Sexually Mature Rats.

Dietary treatment Mg	No. of animals	Initial wt g	Final wt g	Gain per wk g
+	3	156	337*	13.9
—	3	147	249	7.7
+	3	311	385†	14.2
—	8	311	346	7.0
+	4	215	283†	13.6
—	7	209	245	7.0

* Weight after a period of 8 weeks.

† Weight after a period of 5 weeks.

Though the manifestations of magnesium deficiency in the young rat have been well established, the specific effect of magnesium deprivation in the older rat is still questionable. The experiments reported here were designed to determine the effect of insufficient dietary magnesium on sexually mature rats.

Experimental. The composition of the diets used has been reported in a previous paper.⁸ The magnesium low diet contained less than 1.0 mg of magnesium per 100 g, while the control diets contained approximately 30 mg per 100 g. Three different series of rats were used with average beginning weights of 152, 311, and 212 g respectively.

Vasodilatation and hyperemia were not outstanding symptoms in the sexually mature rats on the magnesium-deficient diet. In several animals none was observed at all, but in most instances these symptoms made their appearances 14 to 19 days following the start of the experiment. The vividness of the red color of the ears and extremities during this period was not pronounced, but the slight reddening of these areas was easily detected and usually was observed for 5 to 6 days with a gradual fading towards the end of this period. This increased length of time before vasodilatation and hyperemia could be observed and the lack of marked vasodilatation are differences between the young animals

and the sexually mature animals. It has been shown that young rats on a magnesium-deficient diet show vivid reddening of the ears and extremities within 3 to 5 days after being restricted to the deficient diet.

The animals on the magnesium-deficient diet showed only a slight degree of nervousness and apprehensiveness during the period of vasodilatation and hyperemia, but increased nervousness was noticed 4 to 7 days after vasodilatation had subsided. No cases of spontaneous convulsions were observed.

Significant differences in the average weight gains of the experimental and control animals were noted as early as the second week. By the end of the 5th week the control animals had an average gain of almost twice that of the experimental animals. After 8 weeks on the regimens for the first series and 5 weeks for the second and third series some of the animals on the deficient diet were transferred to the magnesium-supplemented diet. These animals showed an immediate response in rate of gain in weight while those on the deficient diet made only slight gains or lost weight.

After approximately 3 weeks on the experimental diets, edema was observed in the nasal regions of the animals on the deficient diet. The hair became sticky, stiff, and lusterless. Occasionally a sticky, reddish exudate was seen on the feet, noses and ears. Erythematous changes in the skin began to appear between the 23rd and 28th day after the experiment had started. The erythematous changes were characterized by small circular areas on the dorsum of the back or neck in

⁶ Cramer, W., *Lancet*, 1932, **223**, 174.

⁷ Greenberg, D. M., *Ann. Rev. Biochem.*, 1939, **8**, 269.

⁸ Kunkel, H. O., and Pearson, P. B., *Arch. Biochem.*, 1948, **18**, 461.

this medium its multiplication is sufficient to produce measurable quantities of hemagglutinin in the supernatant fluid even when small inocula are employed. It is therefore possible within certain limits to estimate with ease the capacity of the virus to multiply under these conditions. It has also been determined that Influenza A virus (PR8 strain) increases in this type of medium to a level at which its hemagglutinating property can also be readily assayed.

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¹ Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, 1932, **90**, 519.

² Tufts, E. V., and Greenberg, D. M., *J. Biol. Chem.*, 1938, **122**, 693.

³ Watchorn, E., and McCance, R. A., *Biochem. J.*, 1937, **31**, 1379.

⁴ Shrader, G. A., Prickett, C. O., and Salmon, W. D., *J. Nutrition*, 1937, **14**, 85.

⁵ Brookfield, R. W., *Brit. Med. J.*, 1934, **1**, 848.

these points and suggested that the use of the dilute (12.5%) plasma prothrombin time determination was preferred in attempting to evaluate any such changes in man. On the other hand, Poindexter and Meyers³ found that oral administration of aminophyllin to a series of 10 patients had no effect on the plasma prothrombin time. Breyspraak and Greenspan,⁴ in a small series of cases, found that oral and intravenous administration of aminophyllin produced no significant changes in the prothrombin time. Gilbert, Dey, and Trump⁵ performed a series of experiments on both patients and animals and found no alteration in the clotting times, dilute and 100% plasma prothrombin times, or in the response to intravenous injection of heparin following the administration of xanthine drugs.

Material. A single intravenous injection of aminophyllin was given to 18 patients on the wards of the hospital. None had any liver disease or disorder of the blood-forming organs and in no case were drugs being taken which are known to affect the clotting of the blood. Following a control determination of clotting and prothrombin times, each subject was given 0.48 g of aminophyllin by intravenous injection over a 10-minute period. Clotting and prothrombin times were determined 30 minutes and one hour after completing the injection. Moderate tachycardia and diuresis were noted in most cases, but there were no other effects.

It is possible that aminophyllin has a slower action on blood clotting than would be detected following a single intravenous injection. Accordingly, a second group of 12 patients was studied to observe a more prolonged action of aminophyllin on blood coagulation. As in the first group, none of these patients had disease affecting the clotting of the blood nor were any taking drugs with known thromboplastic action.

Following an initial determination of clot-

ting and prothrombin times, each patient was given 0.2 g of aminophyllin, by mouth, 4 times a day for 7 days. Two patients were given plain aminophyllin tablets, but because of nausea the remaining 10 patients took an enteric-coated preparation. At the end of the period of administration, a second determination of clotting and prothrombin times was done. With the enteric-coated preparations no ill effects were noted. Three patients, who had angina pectoris due to coronary insufficiency, obtained marked symptomatic relief while taking the aminophyllin.

Methods. These are described at length because strict attention to technical detail is essential to obtain accurate and consistent results. The clotting time was determined by a modification of the Lee-White method.⁶ The equipment consisted of three 75×10 mm tubes in a water bath maintained at a constant temperature of 36 to 39°C. These tubes were cleaned chemically, rinsed in distilled water, and oven-dried before being used. Venous blood was obtained with as little trauma as possible and was collected in a dry syringe. The tourniquet was released before withdrawal of the blood. The stopwatch was started when one-half the required amount of blood had been withdrawn. One cc of this venous blood was placed into each of the 3 tubes. At 3 minutes the first tube was tilted and this procedure was repeated every 30 seconds until the tube could be tilted to the horizontal without having the blood flow down the side. At that time the second tube was similarly tilted and finally the third tube. The end point was considered to be that point at which the third tube could be tilted to the horizontal without any flow of blood.

The prothrombin time was determined by a slight alteration in the Link-Shapiro modification of Quick's method.⁷ Five cc of venous blood was collected into a chemically clean test tube containing 0.01 g of solid potassium oxalate. After mixing, this sample was centrifuged and the plasma pipetted off and placed in a 75×10 mm test tube. Fifty

³ Poindexter, C. A., and Meyers, L., *Quarterly Bull. Northwestern Univ. Med. School*, 1946, **20**, 130.

⁴ Breyspraak, R. W., and Greenspan, F. S., *Am. J. Med. Sci.*, 1946, **212**, 476.

⁵ Gilbert, N. C., Dey, F. L., and Trump, R. A., *J. Lab. and Clin. Med.*, 1947, **32**, 28.

⁶ Lee, R. I., and White, P. D., *Am. J. Med. Sci.*, 1913, **145**, 495.

⁷ Shapiro, S., *Exp. Med. and Surg.*, 1944, **2**, 103.

most of the animals. This skin condition appeared to become progressively worse with time, and erythema was noticed around the scrotal area in the males, on the abdomen, and on the sides and under the jaws of animals of both sexes. Following the erythema, purpurul skin hemorrhages were observed in the areas which had shown erythema. Extensive eschar formation followed the purpura. Usually the eschars were hard and dry, and the escharotic tissue was confined to small circumscribed areas. Exfoliation followed the escharotic stage, and there was a loss of hair around the areas previously occupied by the eschars. In some cases, ulceration followed exfoliation. When this occurred eschars were usually reformed, and exfoliation generally followed.

Those animals that were transferred to the control diet showed rapid improvement and recovery from the skin lesions. In cases where eschars were present, exfoliation and healing took place rapidly. Denuded areas

began to show hair growth in 7 to 10 days, and these areas could not be detected at the close of the experimental period without close examination which would reveal numerous short hairs. Edema subsided within a week after the animals received the increased dietary magnesium, and the animals appeared in good condition by the close of the experiment.

Summary. Sexually mature rats maintained on a magnesium-deficient diet develop lesions of the skin characterized by erythema, purpurul hemorrhages and eschars. The addition of magnesium to the diet results in recovery from the skin lesions. Other symptoms observed are essentially the same as in the immature animal deprived of magnesium. The increased length of time before the appearance of symptoms in older animals probably reflects increased body stores of magnesium with an increment in the time required to deplete the tissues of this element.

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Effect of Aminophyllin on the Coagulation of Human Blood.*

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Aminophyllin (theophyllin-ethylenediamine) is commonly used in the treatment of patients with heart disease, particularly when the coronary arteries are involved. Recently, emphasis has been put on the importance of intravascular coagulation of the blood on the course and prognosis following coronary occlusion. Observations have been published ascribing to aminophyllin a thromboplastic action. Because of the serious therapeutic implications of this finding, if confirmed, the

present study was undertaken. Field, Larson, Spero, and Link¹ in reviewing the literature, found that a number of authors were of the opinion that the methylxanthines had an accelerating action on blood coagulation. They also reported that single oral doses of a number of methylxanthines produced hyperprothrombinemia and a decreased sensitivity to the action of 3,3'-methylenebis-4-hydroxycoumarin. In view of these findings they believed it possible that use of these drugs in man might produce a tendency toward thrombus formation. Link² again emphasized

* This study was aided by a grant from the Lederle Laboratories, Pearl River, N. Y.

We are indebted to Dr. John W. Fertig, Professor of Biostatistics, Columbia University College of Physicians and Surgeons, for making the statistical analyses.

¹ Field, J. B., Larsen, E. G., Spero, L., and Link, K. P., *J. Biol. Chem.*, 1944, 156, 725.

² Link, K. P., *Harvey Lectures*, 1943-44, 39, 126.

mg of desiccated rabbit lung[†] was placed into 2.5 cc of normal saline solution and stirred for 10 minutes in a water bath at 56 to 58°C. The mixture was then cooled at 26°C for 1 minute. Two and one-half cc of 0.025 molar calcium chloride were added to this thromboplastin suspension and the resulting mixture stirred for an additional 4 minutes. The thromboplastin-calcium chloride mixture was then centrifuged for 5 minutes, the supernatant decanted and placed in 0.2 cc amounts into 75 × 10 mm test tubes.

The actual test was performed by adding 0.1 cc of the plasma to the 0.2 cc of thromboplastin-calcium chloride mixture and stirring the solution vigorously with a wire loop. The stop-watch was started as the first drop of plasma entered the thromboplastin tube. The end point was that time at which a firm clot formed across the wire loop. An identical test was also done using 12.5% plasma. This was obtained by diluting a sample of the plasma 1 to 8 with normal saline solution.

The strength of the thromboplastin was found to vary considerably with different lots of the preparation and for this reason a control plasma prothrombin time was determined on a normal subject at the time each patient was tested. In order to orient the results in relation to a common baseline, the prothrombin times are all expressed as the percentage of the normal control for a given day. Duplicate determinations were made on both undiluted and 12.5% plasma, in the control as well as in the subject whose blood was being studied.

Results. In the group given a single dose of 0.48 g of aminophyllin by intravenous injection there was no significant change in the clotting times. The individual and average variations were slight. The average initial

clotting time was 7.5 minutes. After one-half hour this had increased to 7.7 minutes; the final average was the same, indicating an increase in the clotting time of 0.2 minute (Table I). In the patients on prolonged oral aminophyllin intake there was also very little individual or average variation. The average initial clotting time was 6.0 minutes and the final 7.2 minutes, indicating an increase of 1.2 minutes (Table II).

In the patients who had received aminophyllin by intravenous injection there was little difference in the prothrombin times before and after administration of the drug. The average initial undiluted plasma prothrombin time was 101.8%, at one-half hour it was 102.8%, with a final average of 103.5%, indicating an overall increase of 1.7%. Similar results were obtained using 12.5% plasma. In this case the initial average was 96.8%, with a one-half hour average of 97.4% and a final average of 97.2%. This was a final increase of 0.4%. In the group of patients receiving aminophyllin orally for one week there was again little variation in the prothrombin times before and after drug administration. The initial undiluted plasma determination averaged 96.0% with a final average of 99.9%, indicating an increase of 3.9%. Similarly, in the determination on 12.5% plasma the initial average was 96.4% and the final 98.1%; this was an increase of 1.7%. In none of the results were the differences statistically significant.

Summary. Aminophyllin was given in a single intravenous injection to a group of 18 patients, and orally 4 times daily for a week to a second group of 12 subjects.

There were no statistically significant changes in the clotting time of the blood or in the undiluted or 12.5% (diluted) plasma prothrombin times following the administration of aminophyllin.

[†] Prepared by the Maltine Company, New York.

TABLE I. Clotting and Prothrombin Times Before and After Administration of 0.48 g of Aminophyllin by Intravenous Injection.

Diagnosis	Lee-White clotting time of blood (minutes)				Undiluted plasma prothrombin time (% of normal control)				12.5% plasma prothrombin time (% of normal control)			
	Initial	½ hr	1 hr	Final change	Initial	½ hr	1 hr	Final change	Initial	½ hr	1 hr	Final change
Acute rheumatic fever	7.5	8.5	7.0	- 0.5	100.0	123.5	110.5	+10.5	95.5	95.8	96.6	+ 1.1
Pneumonia	10.0	8.0	9.0	- 1.0	124.1	112.7	106.0	-18.0	84.1	92.3	89.6	+ 5.5
Secondary syphilis	6.5	7.5	7.5	+ 1.0	95.2	112.9	116.3	+21.1	100.2	98.2	107.5	+ 7.3
Diaphragmatic pleurisy	8.0	8.0	10.0	+ 2.0	116.4	107.6	108.7	- 7.7	81.1	79.1	80.1	- 1.0
Lymphadenitis	7.0	7.5	6.5	- 0.5	92.9	98.1	91.1	- 1.8	109.1	116.7	115.3	+ 6.2
Pneumonia	8.0	9.5	10.5	+ 2.5	107.6	100.5	105.9	- 1.7	93.1	98.0	85.1	- 8.0
Coronary insufficiency	9.0	8.5	9.5	+ 0.5	109.0	98.4	96.4	-12.6	104.1	93.4	90.6	- 3.5
Trichinosis	6.5	7.5	6.5	+ 0.0	104.0	98.2	101.3	- 2.7	103.9	101.9	104.8	+ 0.9
Pneumonia	6.5	7.5	6.5	+ 0.0	105.6	101.2	104.3	- 1.3	98.7	101.4	102.1	+ 3.4
Lymphoblastoma	5.5	5.5	5.0	- 0.5	86.1	91.3	95.1	+ 9.0	91.0	97.3	99.2	+ 1.2
Pneumonia	6.5	6.5	7.0	+ 0.5	105.1	111.8	112.7	+ 7.6	97.3	104.9	106.4	+ 9.1
Psychoneurosis	5.0	4.5	4.5	- 0.5	92.3	96.1	91.7	- 0.6	87.7	92.4	93.9	+ 6.2
Pneumonia	7.0	7.0	7.5	+ 0.5	114.8	114.8	124.2	+ 9.4	111.5	100.7	101.4	-10.1
Coronary heart disease	7.0	6.5	6.0	- 1.0	98.8	96.1	106.6	+ 7.6	93.3	100.8	98.6	+ 5.3
Pneumonia	9.5	9.5	10.0	+ 0.5	94.2	95.2	104.7	+10.5	87.4	85.7	85.2	- 9.2
Nasal polyposis	9.0	9.5	9.0	+ 0.0	99.4	96.0	102.8	+ 3.4	98.8	100.8	89.7	- 9.1
Amebic dysentery	8.5	9.0	8.5	+ 0.0	92.7	98.6	89.0	- 3.7	111.6	105.3	104.2	- 7.4
Duodenal ulcer	7.0	7.5	8.0	+ 1.0	93.6	96.4	97.1	+ 3.5	94.7	88.8	90.0	- 4.7

TABLE II.

Clotting and Prothrombin Times Before and After Administration, by Mouth, of 0.2 g of Aminophyllin, Four Times Daily for One Week.

Diagnosis	Lee-White clotting time of blood (min)				Undiluted plasma prothrombin time (% of normal control)				12.5% plasma prothrombin time (% of normal control)			
	Initial	Final	Change		Initial	Final	Change		Initial	Final	Change	
Coronary heart disease	7.5	7.5	± 0.0		102.7	87.0	-15.7		106.2	105.1	- 1.1	
" "	5.5	9.5	+ 4.0		97.2	111.2	+14.0		94.1	99.4	+ 5.3	
" "	5.0	5.5	+ 0.5		107.1	107.2	+ 0.1		91.4	94.2	+ 2.8	
" "	8.5	8.5	± 0.0		109.8	98.2	-11.6		103.4	96.3	- 7.1	
Hypertensive vascular disease	6.0	4.5	- 1.5		91.0	84.3	- 6.7		96.7	91.8	- 4.9	
Cor pulmonale; pulmonary fibrosis	7.0	8.0	+ 1.0		78.7	97.1	+18.4		84.5	88.7	+ 4.2	
Tuberculous pericarditis	5.5	5.5	± 0.0		100.5	100.5	± 0.0		91.4	96.2	+ 4.8	
Coronary heart disease	4.5	8.0	+ 3.5		88.4	87.5	- 0.9		88.4	90.0	+ 1.6	
Thyroid neoplasm	6.0	8.0	+ 2.0		87.9	96.7	+ 8.8		93.1	99.3	+ 6.2	
Hypertensive cardio-vascular disease	6.0	8.0	+ 2.0		113.6	109.8	- 3.8		107.0	118.3	+ 11.3	
Regional enteritis	5.5	7.0	+ 1.5		98.9	113.1	+14.2		116.7	101.7	-15.0	
Coronary heart disease	4.5	6.5	+ 2.0		101.1	105.8	+ 4.7		84.4	96.0	+11.6	

TABLE I.
Treatment of Spontaneous Mammary Cancer Mice with *T. cruzi* Lysate.¹ Animal Strain the Bagg Albino.²

Exp.	Tumor size at start test	Dosage: No. of inject. (subcut.)	Total tumors		% No effect		% inhibition		% Regression		% Deaths	
			Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
A	1 cm or less	45 (total 45 u.)	107	86	69	88	19	8	12	4	50	30
B	>1 cm	45 (total 45 u.)	51	51	86	88	12	10	2	2	71	52
C	1 cm or less	53 (total 15 u.)	37	33	24	54	52	27	24	19	S ⁴	

¹ Controls treated with equivalent volume and dilution of sterile medium.

² Obtained from Mrs. F. O'Grady, Bronx, N. Y. C.

³ Sacrificed 48 hours after 5th injection.

⁴ Sacrificed.

lysate were given to normal mice and a variety of tumor-bearing mice. Only occasional evidence of liver and kidney damage was noted despite the fact that the average survival time of cancerous mice treated with *T. cruzi* lysate was less than the controls injected with sterile medium.

No gross or microscopic differences in the treated tumor-bearing animals of the A strain mice (spontaneous mammary carcinoma¹ and transplanted carcinoma No. 119⁴) were observed as compared with the same types of tumor-bearing mice treated with sterile medium.

This was also true of the spontaneous mammary carcinoma larger than one cm in size of the Bagg-albino strain. (Table I, Experiment B.) However, in this latter strain where the same tumor was less than one cm in size there was a greater degree of inhibition in the treated groups as compared with the control groups. The per cent of these mice showing no effect was: controls 71%, treated 46%. (Combined findings of Experiments A and C, Table I.) This finding was not considered sufficiently significant to state that the lysate of *T. cruzi* exerted a specific cancerolytic

effect. However, the association of this finding with the fact that the survival time of the treated tumor-bearing animals was distinctly less than that of the control tumor-bearing animals does indicate that the lysate may contain some active principle that exerts an effect on tumor-bearing animals, and that this effect was observed only in mice with tumors less than one cm in size. Whether or not this effect is a direct or indirect one is beyond the scope of this study. The differences observed could not be attributed to any nutritional effect since 80% of the animals surviving the experiment showed no weight loss. Those which lost weight were equally divided between treated and control groups, and tumor sizes of all of these latter mice were approximately the same.

In another group of Bagg-albino mice with spontaneous mammary carcinoma one cm or less, 5 daily injections were given, the dose being increased by one unit each day. Forty-eight hours after the last injection, each animal having received a total dose of 15 units, the mice were sacrificed. The results were considered comparable to those obtained in similar mice receiving 45 daily injections (See Table I).

A large series of experiments were performed using mice of the Bagg-albino strain with implanted sarcoma No. 180. This tumor

¹ Obtained from Dr. M. J. Shear, National Cancer Institute, Bethesda, Md.

⁴ Obtained from Dr. T. S. Hauschka, Lankenau Hospital, Philadelphia, Pa.

Preparations of Lysates from Cultures of *T. cruzi* and Their Effects on Normal and Tumor-Bearing Mice.

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(Introduced by A. F. Coca.)

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Klyueva and Roskin^{1,2} have reported the production of an extract of *T. cruzi* which had a specific cancerolytic effect on experimental tumors in mice. Attempts to confirm these findings were unsuccessful.^{3,4,5} The present report is a summary of experiments done in an attempt to verify the findings of the Soviet investigators employing a whole culture lysate.

Materials and Methods. *T. cruzi* cultivation and preparation of *T. cruzi* extracts: Cultures* were grown on NNN agar with an overlay of Tyrode's solution and incubated at 24°-25°C. At the height of growth, the concentration of organisms was determined as follows: A 4 mm loopful of culture was examined at 250× magnification. Fifty fields were examined and the average number of organisms per field divided by two represented the concentration of organisms in millions per ml. (Repeated comparisons with hemocytometer counts confirmed the reliability of this method.) Dilutions were based on units, one unit representing one million organisms.

The cultures were diluted with distilled water and metaphen (Abbot) so that each ml contained 4 units of *T. cruzi* lysate and metaphen in a concentration of 1:10,000. After

storage at 8°C for 24 hours, the trypanosomes were found to have undergone lysis. This was confirmed by microscopic study of the diluted culture which was also examined for sterility. The culture lysates were stored at -20°C until used, except for one experiment in which they were kept at 8°C. None were kept more than 6 days. If not used in this period, the lysate was discarded.

Treatment. These lysates were injected into mice of varying strains and bearing several types of tumors. The injections were always given subcutaneously at a site as far from the tumor as possible. The mice were housed in individual jars and fed a prepared complete pellet diet.

The control animals received injections of sterile medium diluted with the same amount of distilled water and metaphen as the lysates. The number of injections and amounts of solution injected were the same for the control and treated groups.

The following observations were recorded:

(a) Body weight twice weekly.

(b) Tumor size 3 times weekly. (Two diameters were measured by calipers and a drawing representing the area of the tumor was made on the individual records maintained for each mouse.)

(c) Any change in the tumor or in the general condition of the animal was noted.

(d) At necropsy when the animal died or was sacrificed, gross and microscopic examinations of the tumor, liver, kidney, intestine, and heart were made. (Zenker's fixation—H. and E. stain.)

(e) Stool culture studies were made on the Bagg-albino strain of mice used to control subclinical enteric infections. No infections were found throughout the entire series.

Findings. To determine the presence of any toxic factors, injections of the *T. cruzi*

¹ Shimkin, Michael B., Surgeon, U. S. Public Health Service, personal communication, 1947.

² Klyueva, N. G., *Am. Rev. Soviet Med.*, IV, June, 1947, p. 408.

³ Hauschka, T., Saxe, L. H., Jr., and Blair, M., *J. Nat. Cancer Inst.*, 1947, 7, No. 4.

⁴ Hauschka, T., and Goodwin, M. Blair, *Science*, 1948, 107, 600.

⁵ Cohen, A. L., Borsook, H., and Dubnoff, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, 60, 40.

* Obtained from Dr. M. H. Soule, University of Michigan, and designated by us and by the National Inst. of Health, Tropical Disease Division, as the "Soule" or "S" strain.

inflammation.

After many unsuccessful attempts at growing strains of the fibroma virus in tissue culture, Faulkner and Andrewes⁵ finally succeeded in adapting the IA strain to growth in cultures of rabbit testis.

Paschen,⁶ was unable to maintain the IA strain on the chorioallantoic membrane for more than one passage, although the OA strain was still present after 5 passages. However methods and findings are not given in any detail.

Experimental. Methods. The strain of virus employed in most of our experiments was the OA, or tumor-producing strain, obtained from Dr. R. E. Shope as glycerinated rabbit testicle. The IA strain was used similarly in the form of glycerinated rabbit testicle. *Titration*s were carried out by grinding a weighed piece of tissue with sand in a mortar and adding sufficient cold veal infusion broth or saline to make a 10% suspension. Further tenfold dilutions were then made in cold broth, and inoculated subcutaneously in a rabbit in 1.0 cc amounts, no more than 9 tests being carried out in any one animal. The rabbits were observed for appearance of subcutaneous tumors at the site of inoculation. *Neutralization* tests were carried out according to Shope's⁷ method, i.e. a mixture of 1 cc of serum and 1 cc of infection suspension was incubated at 37°C for 1 hour, stored for 2 hours in the refrigerator, then injected in 1 cc amounts into the subcutaneous tissue of a rabbit, together with appropriate controls in the same rabbit.

Results. Adaptation of the Virus to Eggs. The stored glycerinated testicle virus was well washed in cold saline, then ground with sand and saline to make a heavy suspension, which was used to infect a fresh rabbit, 0.5 cc being inoculated into each testicle. Seven days later, when scrotal and testicular edema were pronounced, the animal was killed, small pieces of testicle were ground with sand and

sufficient cold saline to make a 1% suspension by weight. After this suspension had been allowed to settle for about an hour in the refrigerator, 0.1 cc of the suspension was dropped onto the chorioallantoic membrane of 4 12-day-old eggs and was also cultured on a blood agar slant. Three days later, after incubation at 35°C, the membranes were harvested, ground lightly in a pyrex glass grinder, together with 4 cc of saline per membrane. After standing for half an hour the supernatant of this suspension was used as inoculum for a new set of eggs. Passages were made in this fashion at regular 3-day intervals. The membrane suspension from the 7th egg passage produced typical fibroma in a rabbit. The egg passage virus having unfortunately become contaminated at the 8th passage, the testicle from the rabbit injected with 7th passage egg membrane suspension was used as a source of virus. The virus was then maintained in eggs for 18 consecutive passages without mishap. In this second series membranes of the 1st, 5th, 10th, 14th, 15th and 18th passages were found to be infectious for rabbits.

Numerous attempts were made to adapt the virus to growth on the chorioallantoic membrane. In each case where virus from fresh tissues was employed it multiplied in the egg. However glycerinated virus did not always establish itself. In some cases the virus passage was discontinued after only 3 or 4 transfers, in others it was carried through 10 or 12 transfers. It was found that the size of the inoculum could be varied between 0.1 and 0.35 cc, the membranes could be ground in physiological saline, saline buffered with phosphate, veal infusion broth, or even egg fluid, though the latter was less satisfactory. It was, however, found necessary to make passages quite regularly at 3-day intervals. The titer of virus was lower on the 2nd and 4th days than on the 3rd, so that passage at 4-day intervals resulted in loss of the virus within 2 to 3 passages. Eggs which had been incubated for 11 days prior to inoculation sustained the growth of the virus most satisfactorily, regularly yielding membranes which were infectious for rabbits when

⁵ Faulkner, G. H., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1935, 10, 271.

⁶ Paschen, E., *Zent. Bakt.*, 1936, 138, 1.

⁷ Shope, R. E., *J. Exp. Med.*, 1932, 50, 803.

proved to be completely unreliable, both controls and treated animals showing a high percentage of complete regressions. Of a group of 27 mice which received no injections, 19¹ showed complete disappearance of the implanted tumor after the tumor had reached a size of one cm in largest diameter.

These results do not confirm those of Klyueva and Roskin as given in the fragmentary reports available to us. At no time did we have detailed information regarding their methods of *T. cruzi* cultivation and lysate preparation. It is possible that the discrepancy may be due to differences in the ultimate nature of the lysate.

Summary. 1. No inhibition of tumors was noted in Baggb-albino strain mice with spontaneous mammary carcinoma over one cm in size when treated with whole culture lysate of *T. cruzi*; on the other hand in the same mouse strain with tumors under one cm in size a

degree of inhibition was noted which warrants closer study.

2. The inhibition noted could not be attributed to malnutrition effects or concurrent bacterial infections.

3. No inhibition was noted in tumor-bearing "A" strain mice (spontaneous mammary carcinoma; transplanted carcinoma No. 119) when treated with *T. cruzi* whole culture lysates.

4. Survival time of treated tumor-bearing mice was less than that of control treated animals.

5. A simple method is given for a reproducible preparation of *T. cruzi* lysates.

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Propagation of Rabbit Fibroma Virus in the Embryonated Egg.*

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In view of the theoretical interest attached to the relationship of viruses to neoplasms,¹ it seems worth while to report the adaptation to growth in the embryonated egg of a virus which, if it does not cause a true tumor, is at least the etiologic agent of a closely related tumor-like condition. Adaptation of such a virus to a different and relatively simple experimental host such as the developing chick embryo may provide a means of studying the virus-tumor relationship. In fact the earliest use of the chick embryo in the study of viruses was for just this purpose, when

Murphy and Rous obtained growth of a fowl sarcoma on inoculating into the chick embryo either minced tumor tissue or a Berkeley filtrate of such tissue.² The virus studied here is that of rabbit fibroma isolated by Shope³ from a growth on the foot of a wild rabbit. Passed since then in series through domestic rabbits, one strain (the so-called OA strain) has maintained its ability to produce tumors on subcutaneous or intratesticular inoculation. From this original strain there arose a variant (the so-called IA strain described by Andrews⁴) which had lost the ability to produce tumors and caused only

* Part of work reported here was carried out while the author was a National Research Council Fellow in the Medical Sciences.

¹ Rous, P., *Bull. New York Acad. Med.*, 1947, 23, 65.

² Murphy, J. B., and Rous, P., *J. Exp. Med.*, 1912, 15, 119.

³ Shope, R. E., *J. Exp. Med.*, 1932, 56, 793.

⁴ Andrewes, C. H., *J. Exp. Med.*, 1936, 63, 157.

were tested for hemagglutination against red cells from the chicken, duck, mouse, guinea pig, rabbit, horse and sheep; but the membranes from infected eggs produced hemagglutination no more regularly than did those of the uninfected eggs used as controls. Precipitin tests and skin tests in rabbits, using as antigen the allantoic fluid of infected eggs yielded negative findings.

Identification of the Virus Obtained upon Egg Passage. That the virus passed in the egg was actually fibroma virus was shown in 3 ways.

(a) Rabbits inoculated on different occasions with egg passage virus were challenged 2 to 3 weeks later with glycerinated rabbit passage virus and to this they were found to be immune.

(b) A rabbit was immunized by subcutaneous and intratesticular inoculation of egg passage virus and another rabbit was similarly immunized with rabbit passage virus. Both were bled after 14 days and their respective sera used in a neutralization test against fresh rabbit passage virus. Both sera neutralized the virus, no lesion appearing where the mixture was inoculated, whereas areas injected with control mixtures of virus and normal rabbit and virus and saline displayed tumor formation.

(c) A rabbit injected with 8th egg passage fibroma virus was challenged 6 weeks later with a subcutaneous injection of rabbit passage myxoma virus. A control rabbit receiving the same inoculum was dead of myxoma in 10 days, whereas the rabbit which had previously been exposed to egg passage fibroma virus showed no signs of illness. In view of the regularity with which myxoma, virus causes death in normal rabbits it seems certain that the inoculation with egg passage fibroma virus had protected the rabbit against an otherwise fatal infection with myxoma, a phenomenon first described by Shope⁷ in connection with rabbit fibroma.

Growth of the IA Strain of Virus in the Egg. Only 2 experiments were carried out with the IA strain of virus. In both cases the virus was lost at the 4th passage, owing to bacterial contamination. No lesions were observed in

the infected eggs. In the one case in which the virus titer was determined it was only 10^{-2} at the 3rd passage.

Discussion. The experiments described here show that at least one strain (the OA strain) of the virus of infectious fibroma of rabbits can readily be propagated on the chorioallantoic membranes of the developing hen's egg. Although the titers of virus obtained from infected membranes compared favorably with these from rabbit tumor tissue, the virus did not, in our experience, show any tendency to invade the embryo and was not demonstrable in the extra-embryonic fluids. Likewise when virus propagated for a number of passages on the chorioallantoic membrane was injected into the yolk sac it rapidly disappeared. It is possible that virus which had been subjected to a greater number of passages on the chorioallantoic membrane might display greater invasiveness for the egg.

Passage through the embryonated egg did not seem to alter the type of lesion for its original rabbit host. This finding was rather unexpected, in view of the fact that the virus did not produce in the egg the pathological changes which are characteristic in the rabbit, *i.e.* intense fibroblastic proliferation with production of tumor-like masses and, in the wild cottontail rabbit, very striking cytoplasmic inclusion bodies in the overlying epithelium. No such bodies could be found in epithelium covering the chorioallantoic membrane.

Summary. 1. The virus which is the causative agent of infectious fibroma, giving rise to tumor-like nodules in rabbits, was maintained by serial passage on the chorioallantoic membrane of the embryonated hen's egg for 18 consecutive passages.

2. The titer of virus in infected chorioallantoic tissue was slightly lower than the titer in rabbit tissues. It was maintained by using eggs incubated for 11 days prior to inoculation and making passages at 3-day intervals.

3. Invasion of the embryo and extra-embryonic fluids did not take place in eggs inoculated on the chorioallantoic membrane. Inoculation of the virus into the allantoic sac, the amniotic sac, the yolk sac and the embryo itself yielded negative results.

diluted 10^{-4} or 10^{-5} by weight.[†] The titers obtained with 9-day eggs were slightly lower and the titers with 13-day eggs approximately 100-fold lower.

In later passages, whenever possible contamination was feared, penicillin (165 units per egg) and streptomycin (0.0015 g per egg) were added to the membrane suspension.

Viability of Egg Passage Virus. Suspensions of membranes made up in veal infusion broth pH 7.3 to which 20% rabbit serum was added were found to withstand quick freezing with dry ice and alcohol and storage in the dry ice chest for at least 2 months. Suspensions in saline, in egg fluid or even in plain broth were often inactive on thawing. Virus suspensions allowed to stand overnight in the refrigerator showed a roughly 100-fold fall in titer.

Distribution of Virus within the Egg. On several occasions the embryos, yolk sacs, and pooled egg fluids from infected eggs were tested for virus by subcutaneous inoculation into a rabbit. Even when the chorioallantoic membrane suspension proved infectious to rabbits when diluted 10^{-4} or 10^{-5} , no virus was detectable in any other part of the egg.

Routes of Inoculation. Virus which had been through a number of passages on the chorioallantoic membrane was injected in 0.5 cc amounts into the yolk sac. Embryos and yolk sacs harvested 3 to 5 days later contained no demonstrable virus.

Effect of Virus upon the Chick Embryo and its Membranes. In view of the widespread lesions produced on the chorioallantoic membrane by the related myxoma virus,^{8,9} it was a disappointment to find no consistent and recognizable effect of fibroma virus on the chick embryo. Many membranes which were

shown by rabbit inoculation to contain virus in large amounts appeared as delicate in structure as membranes from normal eggs. On the other hand it seemed, in the course of adapting virus from a number of individual rabbits to growth in the egg, as if the strains derived from some rabbits showed more tendency to cause marked edema of the membrane than did other strains. Passages were made in parallel from edematous and from "non-edematous" membranes. Sometimes the edema-producing capacity would remain for a passage or two before fading out. Once it had disappeared it would not reappear in the course of further passages. The nature of this "lesion" is being subjected to further study at the present time.

Sections of a number of membranes were studied histologically. The spindle shaped cells with large nuclei characteristic of fibroma in the rabbit^{3,10,11} could not be demonstrated in the egg. No inclusion bodies were seen. Contrary to the experience reported by Paschen⁶ we were unable to demonstrate elementary bodies with certainty on smears stained with Morosow's stain.

The embryos of eggs infected with fibroma virus appeared normal. Several batches of infected eggs were allowed to hatch. The chicks from these eggs were not tested for presence of virus; however, serum obtained from them at the age of 15 days after hatching failed to neutralize rabbit passage fibroma virus. This lack of detectable immunologic response may well be due to the well-known fact that immature animals respond poorly to antigenic stimuli.

Demonstration of Presence of Virus in the Egg. In the absence of characteristic lesions, the only means of recognizing the presence of virus was back-passage into the rabbit. Quicker methods of recognizing infection in the egg were tried unsuccessfully. The supernates of infected membrane suspensions, both unheated and heated for 40 minutes at 70°C ¹²

[†] Tumor tissue or testicle removed from a rabbit at the height of infection, i.e. about 6 days after inoculation, is usually infectious when diluted 10^{-5} or even 10^{-6} . Thus the titer obtained with egg passage virus even under optimal conditions is not quite so high as in the case of rabbit passage virus.

⁸ Lush, D., *Australian J. Exp. Biol. and Med. Sc.*, 1937, **15**, 131.

⁹ Haagen, E., and Du Dscheng-Hsing, *Zentr. Bakt.*, 1938-39, **143**, 23.

¹⁰ Hurst, E. W., *Australian J. Exp. Biol. and Med. Sc.*, 1938, **16**, 205.

¹¹ Ahlstrom, C. G., *J. Path. and Bact.*, 1938, **46**, 461.

¹² Mills, K. C., and Doehez, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 140.

TABLE I.
Fecal Fat and Nitrogen Excretion Before and After Cholecystectomy.
Means of 10-Day Tests on 8 Dogs.

Condition	Dietary fat g/day	Total fecal fat g/day		% free fatty acids		Fecal N, g/day	
		Mean	t-ratio*	Mean	t-ratio*	Mean	t-ratio*
Normal	53	2.43	—	68.6	—	.96	—
Cholecystectomy	53	1.96	.99	82.4	2.45†	1.09	2.06
	80	2.44	.03	80.9	2.47†	.76	4.13‡

* Post-operative tests paired with normal tests.

† Significant at 5 per cent level of probability.

‡ Significant at 1 per cent level of probability.

unspecified amounts of fat and nitrogen over 48-hour test periods before and after cholecystectomy.⁵

Accordingly, in future experimental attempts to correct steatorrhea due to complete bile diversion, the manner of entry of bile preparations into the intestine, whether in continuous or intermittent doses, should not

alter the results.

Clinically, these results further imply that bile salt replacement therapy and strict limitation of dietary fat are unnecessary as far as fat absorption is concerned in uncomplicated cases of cholecystectomy.

Conclusion. In 8 dogs studied both before and after operation, cholecystectomy produced no significant change in the daily excretion of fat or nitrogen in the feces.

⁵ Balice, G., *Morgagni*, 1928, 70, 835.

16644 P

In vitro Antibacterial Action of Extracts from Coptis Root.

NAI CH'U CHANG. (Introduced by Samuel H. Zia.)

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Recent investigations have demonstrated that higher plants are potential sources for antibiotics,¹ and, specifically, extracts of certain Chinese medicinal plants showed bacteriostatic action.² In view of the great potentialities of such a finding especially in relation to the study of the nature of action of some of the well known Chinese drugs, a detailed study of the root of *Coptis chinensis*, Franch, belonging to the family of Ranunculaceae,³ has been made in the past 7 months. The present paper is a preliminary report of

the antibiotic activity of the dilute alcohol extract of the coptis root against a number of microorganisms *in vitro*.

Method of Extraction. Samples of coptis root of Szechuan variety were obtained mostly from a well established and reputable Chinese pharmacy, Hsi Ho Nien Tang, Peiping, and some directly from Szechuan. The antibiotic activities of all the samples extracted with the method described below were found to be approximately the same. The macerated dried roots were placed in a large flask with 10 parts of 50% alcohol, and set aside at room temperature for 24 hours. At the end of this period the entire suspension was boiled for 30 minutes and filtered while hot through ordinary filter paper. The clear yellowish

¹ Benedict, R. G., and Langlyke, A. F., *Ann. Rev. Microbiol.*, 1947, 1, 193.

² Hsü, C. L., *Nat. Med. J. China*, 1947, 33, 75.

³ Read, B. E., *Chinese Medicinal Plants from Pen Ts'ao Kang Mu*, 1936.

4. Although marked edema of the chorio-allantoic membrane was noted in some of the infected, no pathognomonic lesion, gross or microscopic, was present, so that it was necessary in every instance to resort to back-

passage into rabbits to demonstrate the presence of virus.

5. The type of lesion produced in rabbits by the virus was not significantly altered by repeated passage in hen's eggs.

16643

Effect of Cholecystectomy on Fecal Fat Excretion in Dogs.*

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In a previous report¹ it was demonstrated that diversion of bile from the intestine in dogs results in the failure of absorption of about 60% of fat ingested in the diet. It was also found that oral administration of various bile salt preparations failed to correct this steatorrhea.² One of the questions raised by these results concerned the possible role in fat absorption of the manner of entry of bile into the intestine. It was of interest, therefore, to determine whether cholecystectomy, which converts the normal intermittent flow of concentrated bile into a continuous flow of dilute bile into the intestine,³ would result in a defect in fat absorption.

Methods. Eight normal dogs were placed on a daily diet of 335 g of "Pard" with 40 g added lard, making a total fat content of 53 g. After two days on the diet, feces were collected over a 10-day period and pooled for analyses of total fat and free fatty acids⁴ and for nitrogen (Kjeldahl). The animals were then cholecystectomized and allowed 2 weeks for recovery. Four animals were then replaced on the original diet while the other 4 were given a higher fat diet consisting of 200 g "Pard" with 70 grams added lard

(total fat content, 80 g). Feces were again collected over the last 10 days of a 12-day period, and analyzed. This procedure was next repeated, interchanging the diet for the two groups of dogs. Finally the dogs were sacrificed and autopsy revealed complete removal of the gallbladder and moderate dilation of the biliary passages.

Results. The mean daily excretions of fat and nitrogen in the feces of 8 dogs both before and after cholecystectomy are presented in Table I. The average total fat excretion of 2.43 g per day compares fairly well with the previously reported value of 3.51 g obtained from 5-day tests on 6 dogs and demonstrated to be independent of the fat content of the diet.¹ Cholecystectomy did not significantly alter this rate of fat excretion. Following the operation the percentage of split fecal fat significantly increased; the meaning of this is obscure since further analysis showed that it occurred only during the first experimental period following cholecystectomy. Fecal nitrogen excretion was unaffected by cholecystectomy, although the lower "Pard" content of the high fat diet resulted in diminished nitrogen excretion, due as previously suggested¹ to the reduced content of crude fiber.

Discussion. The present results clearly indicate that there is no impairment in the absorption of dietary fat or nitrogen subsequent to a change from an intermittent flow of concentrated bile to a continuous flow of dilute bile into the intestine. These results confirm a previous study in which 6 dogs were given

* Supported in part by a grant from G. D. Searle & Company, Chicago, Ill.

¹ Heersma, J. R., and Annegers, J. H., *Am. J. Physiol.*, 1948, in press.

² Heersma, J. R., and Annegers, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 339.

³ Puestow, C. B., *Arch. Surg.*, 1931, **23**, 1013.

⁴ Fawcweather, F. S., and Anderson, W. N., *Biochem. J.*, 1946, **40**, 350.

against a number of both Gram positive and Gram negative bacteria. Its lethal dose for albino rats has been found to be more than 250 mg per kg body weight.

The author wishes to express his deep gratitude to Professor Samuel H. Zia for his constant advice and helpful criticisms during this investigation.

16645

Anticoagulant Effect of Dicumarol at Various Prothrombin Levels in Dogs.

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(Introduced by I. S. Ravdin.)

From the Harrison Department of Surgical Research, Schools of Medicine, University of Pennsylvania, Philadelphia.

Since the introduction of dicumarol for clinical use¹ there have been differences of opinion concerning the prothrombin reduction required to produce an adequate anticoagulant effect. Some investigators recommend that the prothrombin level be reduced to 10 to 30% of normal.^{2,3,4} Others state that the effective therapeutic prothrombin zone lies between 20 and 60%;⁵ 35 and 50%⁶ and 50 and 60%.⁷

In 2 reported series of patients in which the prothrombin was reduced to 10 to 30% of normal, hemorrhage occurred in 5%³ and 6%⁴ of the patients. Excessive hypoprothrombinemia (below 10%) occurred in 10 to 20% of patients following doses of dicumarol which usually resulted in 10 to 30% prothrombin levels.⁴ It seems probable, therefore, that the maintenance of higher prothrombin levels might be preferable if the anticoagulant effect is adequate at higher levels.

Little experimental work has been done to clarify this problem. In the experiments herein reported the anticoagulant effect of

dicumarol at various prothrombin levels in dogs has been studied in order to determine whether there is a definite and consistent correlation between prothrombin levels and anticoagulant activity.

Method. The dogs were anesthetized with intravenous sodium pentobarbital, and the femoral artery was exposed and cannulated with as little trauma as possible. Thrombosis was produced by inserting into the femoral artery a sterile glass cannula 1.0 mm in diameter at its constricted opening. The cannula widened out to a 3.5 mm diameter where it was connected to a mercury manometer by sterile rubber tubing. The entire system was filled with sterile physiological saline solution. Additional saline was introduced through a T side-arm in the tubing to produce a positive pressure approximately equal to the mean arterial blood pressure in order to prevent the passage of blood into the tubing. When thrombosis occurred, the mercury column in the manometer ceased oscillating, and this was taken as the end-point.

The prothrombin level of each dog was determined within two hours of the time it was cannulated by the Quick method, with the plasma diluted 50% with normal physiological saline solution. A normal curve was plotted each day the experiment was performed to eliminate errors due to a variation in the potency of the thromboplastin. Table I expresses the prothrombin times (in seconds) of various percentages of normal plasma (50%

¹ Allen, E. V., Barker, N. W., and Waugh, J. M., *J.A.M.A.*, 1942, **120**, 1009.

² Barker, N. W., *Minnesota Med.*, 1944, **27**, 102.

³ Allen, E. V., *J.A.M.A.*, 1947, **134**, 323.

⁴ Cosgriff, S. W., Cross, R. J., and Habib, D. V., *Surg. Clin. N. Am.*, April 1948, 324.

⁵ Levam, J. B., *Ann. Int. Med.*, 1946, **25**, 941.

⁶ Peters, H. R., Guyther, J. R., and Brambel, C. E., *J.A.M.A.*, 1946, **130**, 398.

⁷ Cummine, H., *M. J. Australia*, 1947, **34**, 302.

amber filtrate was then evaporated to dryness. The dried crude extract was yellowish brown in color and very bitter in taste. Its aqueous solution was used for various tests to be described.

Method of Assay. Series of 2-fold serial dilutions of the aqueous extract solution were made in 1% serum broth in small test tubes, and tubes of each series were seeded with one loopful of 18-hour broth culture of one of the many types of organisms to be tested. The tubes, after being shaken, were incubated at 37°C and readings were made after 24 hours incubation. The end point of bacteriostasis was taken as the highest dilution in which growth in the tubes was completely inhibited and subsequent cultures on blood agar plates still showed growth. Bactericidal power was determined by the absence of growth on subculture on blood agar plates.

Results. The effectiveness of the crude extract of the coptis root in inhibiting cultures of 26 types of bacteria is shown in Table I.

TABLE I.
Inhibition Power of the Crude Extract of the
Coptis Root on Bacteria.

Organism	Max. inhibiting dilution
<i>Staph. aureus</i> (2 strains)	1:3200 -1:6400
<i>Staph. citreus</i> (2 strains)	1:6400 -1:12800
<i>Staph. albus</i>	1:12800
<i>Strep. beta hemolyticus</i>	1:3200
<i>Strep. alpha hemolyticus</i>	1:3200
<i>Strep. gamma</i>	1:3200
<i>C. diphtheriae</i>	1:3200 -1:6400
Diphtheroid	1:3200
<i>Klebsiella pneumoniae</i>	1:1600
<i>B. subtilis</i>	1:6400
<i>Micrococcus tetragenus</i>	1:12800
<i>Vibrio comma</i>	1:3200
<i>Shigella dysenteriae</i> (5 strains)	1:400 -1:12800
<i>Shigella sonnei</i>	1:800
<i>Shigella paradysenteriae</i>	1:800
<i>Br. abortus</i>	1:25600-1:51200
<i>Br. melitensis</i>	1:3200
<i>Eberthella typhosa</i>	
H901	0
O901	0
<i>S. paratyphi</i>	0
<i>S. schottmuelleri</i>	0
<i>S. enteritidis</i>	0
<i>E. coli</i>	0
<i>Proteus</i> OX ₁₉	0
<i>Pseudomonas pyocyanea</i>	0
<i>Serratia marcescens</i>	0

0 indicates no inhibition in 1:200.

From Table I, it seems very clear that the crude extract of the coptis root in high dilutions inhibited the growth of all Gram positive organisms tested and also that of certain Gram negative ones. The extract appeared particularly effective against *Br. abortus*, as, even in a dilution of 1:25600, growth of this organism was inhibited. Of some interest is that one strain of *Staphylococcus citreus*, isolated from a patient with carbuncle, and highly resistant toward the action of penicillin (50 U/ml) showed great susceptibility to the crude extract in the dilution of 1:12800. When subcultures were made on blood agar plates, it was shown that the bacteriostatic power of the extract was usually 2-4 times greater than its bactericidal power.

Tests for Toxicity. Adult albino rats of 200 g body weight were injected intraperitoneally with 5% aqueous extract solution and observed at intervals of 10 minutes, 30 minutes, 1 hour and 24 hours after injection. Amounts of 2 and 1.5 ml caused death of the animals in 30 minutes, while those of 1 and 0.5 ml produced no obvious toxic symptoms. The animals remained well in 1 week.

Discussion. The root of coptis has been generally used as a bitter tonic, but, in China, it has been extensively used in the treatment of dysentery since ancient times.¹ Results of *in vitro* tests made in the present study extended those of the previous worker in demonstrating bacteriostatic power against various types of dysentery bacilli and may explain why coptis root has been used empirically by the Chinese. The unexpected finding of the special effectiveness of this extract against our stock strain of *Br. abortus* but not particularly so against *Br. melitensis* requires further study. It may be mentioned that the active principle so far has been found to be completely adsorbed on activated charcoal. Further investigations regarding purification and chemical analysis and *in vivo* tests of this extract are being undertaken.

Summary. The dilute alcohol extract of the coptis root has been demonstrated to have a definite and fairly high antibacterial action

¹ Li, S. C., Catalogue of Native Herbs, A.D. 1596.

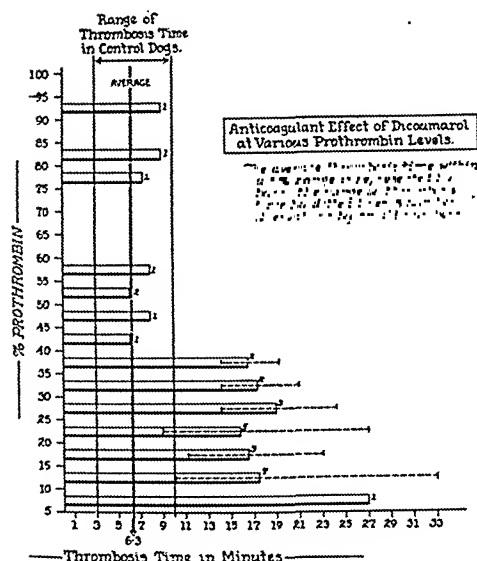


FIG. 2.

bosis, as compared with clinical thrombosis. Neither is it an *in vitro* method, as shown by the lack of correlation between the Lee-White coagulation time and the cannula "thrombosis time". For the purpose sought in this experiment of obtaining a comparative quantitative estimate of the anticoagulant activity of dicumarol at various prothrombin levels, the method has appeared adequate. The method of producing thrombosis used in this experiment obviously does not simulate clinical conditions in which thrombosis occurs. A more suitable method of producing experimental thrombosis has not been devised so far as we know.

The two striking results in the experiment are: (1) the sharp line of demarcation of anticoagulant activity at a prothrombin level

of 40% and (2) the apparent uniformity of the average anticoagulant activity at 10, 20, 30 and 40% prothrombin levels.

It is to be emphasized that under the conditions of these experiments the response to dicumarol in dogs may bear little relationship to dicumarol response in man. Yet it is apparent that in dogs: (1) prothrombin levels above 40% had no demonstrable effect upon prolongation of the thrombosis time and (2) prothrombin levels below 40% did significantly increase the thrombosis time in 20 of 23 experiments.

In view of these results and the lack of conclusive experimental evidence elsewhere, the question must be raised as to whether the clinical use of prothrombin levels of 10% to 30% may be too low or whether levels of 40% to 60% may be too high for effective yet safe anticoagulant therapy.

As to the apparent uniformity of anticoagulant activity at 10, 20, 30 and 40%, this may merely reflect a limitation of the sensitivity of the method or else may reveal that an all or none, rather than a mass action phenomenon, is associated with the method. This cannot be definitely answered at present from the data available.

Conclusions. 1. The anticoagulant effect of dicumarol at various prothrombin levels in dogs has been studied.

2. In control animals thrombosis occurred, on the average, in 6.3 minutes, with a variation from 3 to 10 minutes.

3. In dicumarolized dogs the time of thrombosis was not delayed at prothrombin levels above 40%. Below 40% thrombosis occurred on the average, in 16.6 minutes.

TABLE I.
Plasma Prothrombin Times (in Seconds) of Normal Healthy Dogs.

Percentage dilution of normal control plasma (50% diluted)	Total No. of determinations	Avg time (sec)	Range of times (sec)
100	95	9.9	8.2-11.5
90	18	10.2	9.0-11.5
80	21	10.3	9.0-11.8
75	35	10.8	9.4-12.9
60	31	11.0	9.6-13.9
50	55	11.3	9.8-14.8
40	39	12.4	10.0-17.6
30	43	14.0	10.5-19.8
25	36	14.7	12.7-21.6
20	51	17.1	12.3-26.1
15	33	18.7	15.3-32.3
10	47	27.6	18.2-50.7
5	8	38.0	35.9-41.0

diluted) and shows the averages and ranges obtained.

Results. In a control series of 14 normal animals the femoral arteries were cannulated 22 times. Thrombosis occurred, on the average, in 6.3 minutes, with a variation from 3 to 10 minutes (Fig. 1).

The experimental series consisted of 32 dogs ranging in weight from 8.0 kg to 14.2 kg. Dicumarol in doses from 10.0 mg to 400 mg was administered orally 72 hr before the experimental procedure. Prothrombin levels ranging from 95% to 8% of normal were obtained following this therapy.

Prolongation of the "thrombosis time" was found to occur at prothrombin levels of 40% and below (Fig. 2). Of 23 dogs with levels below 40%, the average time was 16.6 minutes. In 20 dogs the thrombosis time was prolonged beyond the longest time of any in the control series. In the remaining 3 thrombosis occurred (10, 10 and 9 minutes) at about the same time as the longest in the control series (10 minutes). The average prolongation of the thrombosis time did not vary significantly at 10, 20, 30 and 40% prothrombin levels.

Prolongation of the "thrombosis time" was not found to occur in dogs with prothrombin levels of 44% or higher. All of the nine animals in this group had "thrombosis times" within the limits of the controls, the average being 7.2 minutes.

In 10 of the dicumarolized animals with prothrombin levels from 8 to 95%, there

was no correlation between the Lee-White coagulation time and the cannula "thrombosis time".

Comment. Advantages of the method used are that: (1) the endpoint is clear cut; (2) thrombosis occurs in a relatively short period of time; and (3) the time of occurrence of thrombosis in control animals is relatively constant. Since thrombosis occurred in or about the cannula, this is not strictly an *in vivo* method of producing throm-

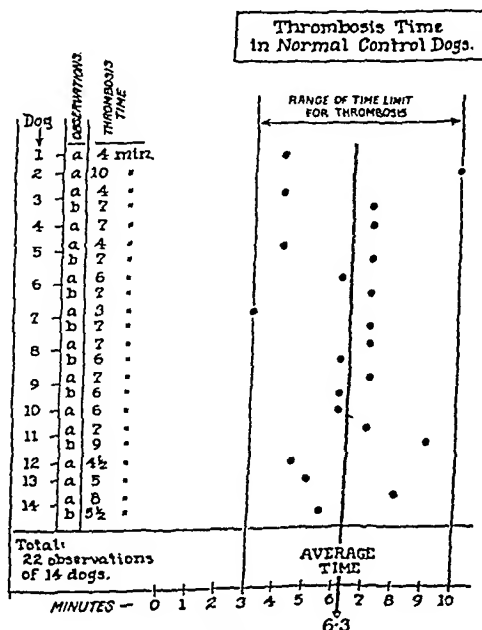


FIG. 1.

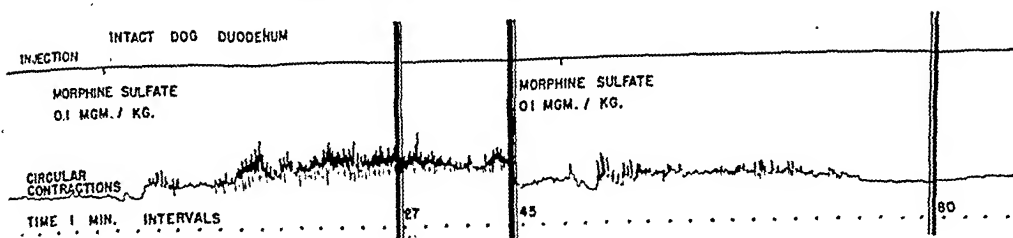


FIG. 1.

The effect of repeated equal doses of morphine sulfate on the circular contractions of the duodenum of the anesthetized dog. The drug was injected intravenously at the points indicated on the injection line.

lowed with about 2 cc of physiological saline. Controls using the saline alone showed that the spontaneous intestinal contractions were not altered by this volume of saline.

Results. Early in the progress of the experiments it was found that repeated equal doses of morphine failed to produce equivalent responses in the intestine. Although the muscular responses would be qualitatively similar the intensity and duration of these responses were much diminished with each additional injection. Fig. 1 demonstrates this tachyphylactic type of phenomenon which occurs in the response of the circular muscle when an intravenous dose of morphine of 0.1 mg per kg is given and then repeated after the activity had returned to normal. After this phenomenon had been repeatedly demonstrated all subsequent experiments were conducted so that the animal received only one injection of morphine.

Two principal types of response to the drugs used were obtained. One of the types of response was an increased level of tonus with either an increase or no change in the frequency and amplitude of the normal spontaneous contractions. This type of response is referred to in this paper as "increased activity." The second type of muscular response was a decreased level of tonus and either a decrease or no change in the frequency and amplitude of the normal spontaneous contractions. This type of response is referred to as "decreased activity."

Table I summarizes the gross qualitative responses obtained on a series of experiments conducted on 28 dogs. In all experiments acetylcholine was used as a control drug. The table shows that 0.05 mg per kg of this compound, given intravenously, produced an in-

creased activity of the circular muscle in all but one of 25 experiments. In simultaneous records this type of response occurred in the longitudinal muscle in 18 of the 25 experiments. In one of the experiments both the circular and longitudinal muscle records showed decreased activity. In 5 of the experiments the longitudinal muscle showed decreased activity while the circular muscle responded by an increased activity.

A dosage range of morphine of from 0.01 mg per kg to 1.0 mg per kg was used. The smallest dose of morphine which was consistently effective in producing an alteration in the intestinal muscular activity was 0.05 mg per kg. Fig. 2 shows the type of record obtained. A similarly equivalent qualitative response was obtained in five such experiments. Table I summarizes the response obtained with 0.1 and 0.5 mg per kg of morphine in the series of seventeen experiments on seventeen different dogs. The table shows that the circular muscle responded with an increased activity in 16 of the 17 experiments. In 13 of the experiments the longitudinal muscle showed either no significant response or a decreased activity. With a dose of morphine of 0.5 mg per kg the response of the circular muscle was that of increased activity in all of the 17 experiments. In contrast to this, the longitudinal musculature showed increased activity in only two of the 17 experiments, whereas in 10 of the experiments the result was that of decreased activity and in 5 experiments there was no observable alteration in muscle activity.

One mg per kg of morphine produced an increased activity in the circular muscle in 6 experiments, and in 5 of the 6 experiments a decreased activity was the response recorded

Response of the Duodenum to Morphine.

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The earliest investigators concerned with the response of the intestine to morphine were predominantly of the opinion that this drug produced a relaxation of the muscle of the intestine. More recent investigations have changed this trend of belief by showing that morphine produced an increased pressure within the intestine and increased movements of various segments of the intestine as measured by one or more forms of a balloon method.

It has been pointed out by Krueger¹ and emphasized by Quigley, Highstone and Ivy² that erroneous conclusions may be drawn concerning the sum total effect of a drug on the intestinal muscle by using a method which involves inserting a balloon in the lumen of the intestine and recording activity in terms of changes in volume of the balloon. Thus in an attempt to improve the experimental procedure, bolus methods² and multiple balloon methods³ have been devised and used.

The present study is concerned with the muscular response of the duodenum to morphine as measured by a method which gives simultaneous graphic recordings of both the circular and longitudinal muscle activity in a given segment of the duodenum in the intact anesthetized dog.

Methods. The circular contractions of the duodenum were recorded by the method of Krop and Loomis.⁴ The balloon inserted in the duodenum was constructed so that its length was not more than 2 cm. Such a balloon would come in contact with about 1 cm length of intestinal mucosa when fully ex-

panded. Observations with this apparatus *in situ* showed that longitudinal movements of the intestine did not record through the manometer. Circular contractions, on the other hand, compressed the balloon and were recorded as changes in the level of the fluid in the manometer. The longitudinal contractions of the duodenum were recorded by the internal organ apparatus described by Jackson.⁵ Observations with this apparatus *in situ* showed that the records obtained were predominantly those of longitudinal muscle activity.

Mature mongrel dogs in good health were used throughout the experiments. The dogs were anesthetized with 35 mg per kilo sodium pentobarbital given intravenously. A midline abdominal incision from the xiphoid process of the sternum to about 2 cm above the pubis was made. The intestine was retracted to one side, an incision was made in the pyloric end of the stomach on its anterior surface and the balloon was inserted through the incision into the stomach, directed through the pyloric sphincter and into the duodenum so that it lay about 5 cm distal to the sphincter. Jackson's internal organ apparatus was then sutured to the mucosal surface of the segment of duodenum which contained the rubber balloon within its lumen. The intestine was then replaced in its approximate normal position and the cut edges of the abdominal incision were approximated and sutured.

All injections were given intravenously through one of the superficial branches of the femoral vein. The drugs were dissolved in physiological saline. The volume of each injection was always less than 5 cc and the solutions were maintained at a temperature of 38°C. Each injection of a drug was fol-

¹ Krueger, H., *Physiol. Rev.*, 1937, 65, 618.

² Quigley, J. P., Highstone, W. H., and Ivy, A. C., *J. Pharm. and Exp. Therap.*, 1934, 51, 305.

³ Krueger, H., *J. Pharm. and Exp. Therap.*, 1934, 51, 440.

⁴ Krop, S., and Loomis, T. A., *Science*, 1945, 102, 155.

⁵ Jackson, D. E., *Experimental Pharmacology and Therapeutics*, C. V. Mosby Co., 1939, page 89.

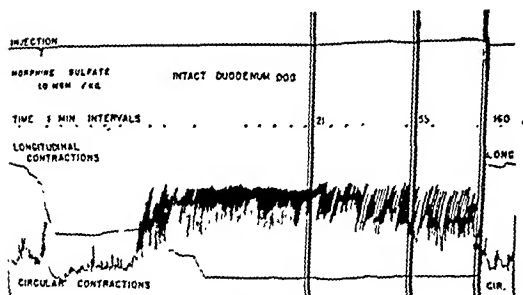


FIG. 3.

The "increased activity" of the circular muscle and the simultaneous "decreased activity" of the longitudinal muscle of the intact segment of the dog duodenum as it is produced by a dose of 1.0 mg/kg of morphine sulfate.

ished by the subsequent administration of morphine.

Atropine only partially decreased the increased activity in the circular muscle produced by morphine. Atropine also abolished any activity present in the longitudinal muscle group when given following the injection of morphine.

In 3 experiments in the physostigminized animal, morphine produced a response of a type similar to that most frequently produced in the normal animal. This was a decreased activity of the longitudinal muscle and an increased activity of the circular muscle (Fig. 7). However, the mean duration of the response to morphine (0.1 mg per kg) in the eserinizated animal was 222 minutes (3 experiments) whereas the maximum duration of response obtained in the normal dog to 0.1 mg per kg of morphine was 110 minutes (17 experiments).

Discussion. The evidence in the literature at the present time indicates that morphine does not produce a consistently specific type of effect on smooth muscle throughout various parts of the gastrointestinal tract.^{1-3,6-9,11-13} Furthermore, reports concerned with the effect of morphine on the atropinized or eserinizated intestine are not consistent in the same or different animals.^{7,10,14}

⁶ Trendelenberg, P., *Arch. F. Exp. Path. u. Pharmacol.*, 1917, **81**, 55.

⁷ Veach, H. O., *J. Pharm. and Exp. Therap.*, 1937, **61**, 230.

⁸ Plant, O. H., and Miller, G. H., *J. Pharm. and Exp. Therap.*, 1928, **32**, 437.

Acetylcholine and barium chloride consistently produced increased activity in both the circular and longitudinal muscle layers with the experimental arrangement described in this paper. Atropine sulfate consistently produced decreased activity of both of these muscle layers. The most consistent response produced by morphine injected intravenously was that of an immediate increased activity of the circular muscle layer and a decreased activity of the longitudinal muscle layer. This type of response was marked and prolonged and records always showed a return to normal when allowed sufficient time. Occasionally there occurred an immediate very brief (5 minutes or less) interval of little change or an increased activity of the longitudinal muscle or a decreased activity of the circular muscle following the injection of morphine.

Summary and conclusions. 1. The phenomenon of tachyphylaxis of the intestinal musculature to morphine was demonstrated.

⁹ Plant, O. H., and Miller, G. H., *J. Pharm. and Exp. Therap.*, 1928, **32**, 413.

¹⁰ Plant, O. H., and Miller, G. H., *J. Pharm. and Exp. Therap.*, 1926, **27**, 361.

¹¹ Krueger, H., *J. Pharm. and Exp. Therap.*, 1934, **51**, 85.

¹² Gruber, C. M., and Pipkin, G., *J. Pharm. and Exp. Therap.*, 1930, **38**, 401.

¹³ Wilen, C. J., and Dragstedt, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 1056.

¹⁴ Gruber, C. M., Greene, W. W., Drayer, C. S., and Crawford, W. M., *J. Pharm. and Exp. Therap.*, 1930, **38**, 389.

TABLE I.

Summary of Frequency and Type of Response of Duodenal Muscle to Acetylcholine, Morphine, Barium, and Atropine. Maximum and minimum duration of response is presented rather than mean and standard error, since accurate determination of duration of time before the record returned to normal was indefinite within limits of approximately 10 minutes.

1. No. of experiments	0.05 mg/kg Acetylcholine		0.1 mg/kg Morphine SO ₄		0.5 mg/kg Morphine SO ₄		1.0 mg/kg Morphine SO ₄		1.0 mg/kg Barium Chloride		2.0 mg/kg Atropine SO ₄	
	Longitudinal muscle	Circular muscle	Long. m.	Circ. m.	Long. m.	Circ. m.	Long. m.	Circ. m.	Long. m.	Circ. m.	Long. m.	Circ. m.
2. Increased activity (number of exper. showing:)	25	25	17	17	17	17	6	6	5	5	3	3
3. Decreased activity	18	24	4	16	17	17	0	6	5	5	0	0
4. No significant response	5	1	6	1	10	0	5	0	0	0	3	3
5. Range of duration of response (min)	2-16	2-9	40-110	40-100	30-160	40-160	60-200	60-280	—	—	—	—

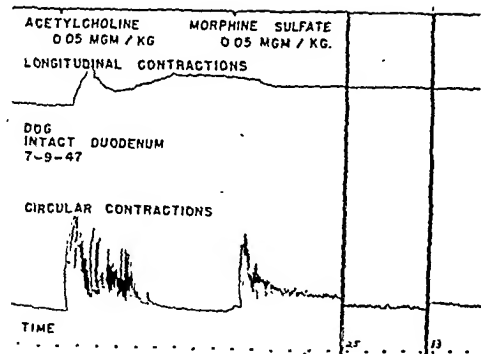


FIG. 2.

The "increased activity" of the circular muscle and simultaneous "decreased activity" of the longitudinal muscle produced by 0.05 mg per kg of morphine sulfate following a control acetylcholine injection. Time is recorded as dots at one minute intervals.

by the longitudinal muscle apparatus. Fig. 3 is a typical record of this result.

These results show that the type of response was essentially independent of the dose of morphine used over the range which was studied. The duration of the response was similar in both the circular and longitudinal groups (Table I) for a given dose of morphine. The duration of response generally was greater with higher doses than with the lower doses over the range studied.

Barium chloride in a dose of 1.0 mg per kg consistently produced an increased activity in both the circular and longitudinal muscle records. When morphine is given at the time when a maximum response to barium is present generally little or no alteration in the activity of the duodenal muscle occurs.

Atropine repeatedly produced a decreased activity in both the circular and longitudinal muscle of the duodenum. A dose of morphine of 0.1 mg per kg produced an increased activity in the circular muscle in the atropinized animal at a time when a control acetylcholine injection produced no response. The response of the longitudinal musculature in an atropinized animal was indefinite since atropine frequently produced such a profound decreased tonus and often completely abolished all activity of the muscle. However, when slight activity remained in the longitudinal muscle after administration of atropine, it was abol-

the two groups were not significantly different. The Dibenamine treated animals showed slightly higher blood flow values throughout, with faster pulse rates. Vasomotor resistance values, while showing roughly parallel changes in the two groups, were consistently lower in the treated dogs. There was no demonstrable correlation between the dilution of the blood and the survival period. In fact, as a group, the treated animals showed less dilution than the controls.

It has been shown that animals subjected to contusion of the muscles of both hind legs, by multiple blows with a light mallet, show higher resistance values than after hemorrhage,⁶ and die after a smaller blood volume loss than would be required by hemorrhage.⁷ This higher resistance is presumably brought about by afferent nervous discharge from the traumatized limbs.⁸ Nine control animals, under morphine-nembutal anesthesia, were given 80 blows/kg b.w. to each hind leg. All these dogs died, with the average survival of 3½ hours. Nine more dogs were given Dibenamine, as above, 1 hour before the

trauma. Eight of these recovered, the ninth dog surviving 15 hours. As with the hemorrhage experiment, pressure values were not different in the two series. Pulse rates of the Dibenamine-treated animals were greater, and flows were maintained at higher levels. They therefore showed a lower vasomotor resistance.

A limited number of blood volumes, determined by T-1824 injection, indicated that the blood volume loss in these animals was sufficient in itself to have caused death. The blood volume loss was of similar magnitude in treated and untreated dogs. Peripheral resistance was definitely higher than after hemorrhage. The treated dogs did not show this rise in resistance, but rather a vasodilation. Death in the control series may be preceded by the appearance of cardiac irregularity, which developed while the peripheral resistance was still high. Both groups showed a hemoconcentration. In the control series, however, this developed not during the trauma but after its completion.

Summary. A hemorrhage of 30 cc/kg proved fatal in 93% of the cases. Pre-treatment with Dibenamine reduced this mortality to 10%. Pulse rate and blood flow per second were consistently greater in the second series. Administration of 80 blows/kg to each hind leg with a light mallet produced fatal shock in 100% of the cases. Pre-treatment with Dibenamine reduced the mortality to 11%. Control animals showed vasoconstriction, treated animals vasodilation.

⁶ Root, W. S., Walcott, W. W., and Gregerson, M. I., *Am. J. Physiol.*, 1947, **151**, 34.

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2. The most consistent response of the circular muscle of the duodenum to morphine was an increase in the many phases of muscular activity. This includes elevation of the tonus level with increased frequency and amplitude of the normal spontaneous tonic contractions.

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16647 P

Protective Action of Dibenamine After Hemorrhage and After Muscle Trauma.*

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Wiggers and associates^{1,2} have demonstrated that dogs given the sympatholytic substance Dibenamine showed a higher survival rate than controls from shock produced by hemorrhage. The inference made is that abolition of a severe vasoconstriction normally following hemorrhage protects the life of the animal.

In a series of dogs under morphine-nembutal anesthesia, 6 arterial hemorrhages of 5 cc/kg b.w. each, were performed at 10 minute intervals. Aortic pressure pulse contours were recorded during the hemorrhage period, and for an additional hour. All wounds were then closed, and the animals returned to their cages. Cardiac outputs were calculated from the pressure pulse contours,³ and in a selected series by the dye injection method,⁴ and vaso-motor resistance (Rv) calculated as the mean aortic pressure less 20 mm Hg divided by the flow per second.⁵ Specific gravity values of

plasma and whole blood were determined at 10 minute intervals by the falling drop technique.

This experiment was done during the summer months, so that an expectedly sub-lethal hemorrhage proved fatal in 13 of 14 dogs, with an average survival of the 13 dogs of 4 hours. Ten more dogs were given 10-20 mg/kg Dibenamine,[†] in 50 cc saline, i.v., one hour before the hemorrhage was begun. The completeness of the sympathetic block was demonstrated by the injection of epinephrine 5 minutes before the first bleeding. Nine of these animals survived. To rule out the possible effect of the salt solution itself, the last 5 of the control series received 50 cc saline one hour before the hemorrhage. All of these dogs died.

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The second group of data, the selected series, consisting of clearance periods chosen on the basis of plasma equilibrium and technical perfection, but, of course, without regard to mannitol recovery, contains 30 periods from 16 tests on 12 patients.¹

This series contains only periods preceded and followed by determinations of plasma concentration that did not differ by more than 3.5 mg%, a value within the error of the method (standard deviation = ± 2.5 mg%). An actual difference of this magnitude, in 2 specimens drawn 30 minutes apart, would represent, on the basis of an average normal mannitol space of 15 liters,² a gain or loss of 17.5 mg/min. and thus might obscure a very large extra renal loss. To determine whether equilibrium had been obtained in these periods, they are further subdivided into 3 subgroups: those periods with bracketing plasma levels differing by less than 1 mg%, those with levels falling 1 to 3.5 mg%, and those rising 1 to 3.5 mg%. If equilibrium had not been established one would expect an excessive urinary recovery in the periods with falling levels and a deficient recovery in those

with rising levels. Eighteen periods were bracketed by levels differing by less than 1 mg% and the mean out-in ratio of these is 0.990. There were 8 periods in the falling group with a mean ratio of 0.995 and 4 in the rising group with a mean ratio of 0.970. Since, if these values represented actual differences in plasma concentration, the ratios would be 117 and 0.82 for the falling and rising groups respectively we feel that equilibrium had been established. In these selected periods, an average of 99.3 mg of mannitol was excreted for every 100 mg injected (range 87-114). This small series has a standard deviation of 6.0 with a standard error of the mean of 1.1, giving for the difference 100.0-99.3 a *t* value of 0.64, thus failing to establish a difference between the urinary recovery of mannitol and the amount injected.

Summary and Conclusions. 1. In 92 unselected clearance periods in 29 tests on 22 patients 100.4 mg of mannitol were recovered in the urine for every 100 mg injected.

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16649

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Is Mannitol Metabolized?*

JOHN KAPP CLARK¹ AND HAROLD G. BARKER.² (Introduced by Carl F. Schmidt.)*From the Departments of Pharmacology and Medicine, and the Harrison Department of Surgical Research, University of Pennsylvania School of Medicine and Hospital, Philadelphia, Pa.*

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of one hour, when possible, was allowed for equilibration, urine was collected by catheter with bladder washing and clearance periods were usually 20 minutes or longer. Plasma levels varied from 100 to 50 mg%. Mannitol was determined by a modification⁶ of Smith's method⁷ in most cases and by the colorimetric method⁸ in a few. Total para-aminohippurate was determined by a modification of Smith's method.⁹

Results. Our data are presented in two groups. The first, the unselected series, consists of all patients in whom both urine and infusion solutions were analyzed and contains 92 clearance periods in 29 tests on 22 patients with relatively normal renal function.⁸

The average urinary recovery of mannitol for all 92 periods was 100.4 mg for every 100 mg injected (range 62 to 156). The standard deviation of this series is 16.7; standard error of the mean 1.74. For the difference 100.4-100.0 the t value is 0.23, indicating that the amount recovered in the urine does not differ significantly from that injected.

In many of the clearances satisfactory mannitol plasma equilibrium was not achieved due to inaccurate preclearance estimation of renal function, a priming dose of improper size, changing renal function, etc. In addition, some periods are inaccurate due to technical difficulties such as obvious urine collection errors, chemical analytical errors, pump

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ERWIN LEVIN, JOSEPH B. KIRSNER, AND WALTER L. PALMER.

From the Albert Meritt Billings Hospital, Department of Medicine, University of Chicago Clinics.

A recent study^{1,2} demonstrated that the volume, concentration, and output of acid in the nocturnal gastric secretion are usually

¹ Levin, E., Kirsner, J. B., Palmer, W. L., and Butler, C., *Arch. Surg.*, 1948, 46, 345.

² Levin, E., Kirsner, J. B., Palmer, W. L., and Butler, C., *Gastroenterology*, 1948, 10, 952.

higher in patients with duodenal ulcer than in normal healthy individuals. The patients had been admitted to the hospital because of ulcer distress. Several observers have suggested that the presence of symptoms may be a contributing factor in causing hypersecretion in duodenal ulcer patients. It

Is Mannitol Metabolized?*

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If, as has been recently suggested,^{1,2} mannitol is in part disposed of by non-renal means, probably metabolic, it cannot be used as a measure of extracellular fluid space.³ Such a circumstance would render invalid calculations of glomerular filtration rate in which the rate of infusion is substituted for the rate of excretion, UV/P , in the clearance formula $C = UV/P$ after the plasma level, P , has become constant,⁴ or from the falling curve of plasma concentration without urine collection,⁵ if mannitol is used as a test substance. Since the publication of the constant infusion technic for determining renal clearances,⁴ we have routinely infused our test solutions, mannitol and para-aminohippurate, from a syringe with a motor driven worm screw, and our data on subjects with relatively normal renal function, in cases where both infusion solutions and urine were analyzed, indicate no extra-renal loss when mannitol recovered in the urine is balanced against that infused. In these tests, a period

of one hour, when possible, was allowed for equilibration, urine was collected by catheter with bladder washing and clearance periods were usually 20 minutes or longer. Plasma levels varied from 100 to 50 mg%. Mannitol was determined by a modification⁶ of Smith's method⁷ in most cases and by the colorimetric method⁸ in a few. Total para-aminohippurate was determined by a modification of Smith's method.⁹

Results. Our data are presented in two groups. The first, the unselected series, consists of all patients in whom both urine and infusion solutions were analyzed and contains 92 clearance periods in 29 tests on 22 patients with relatively normal renal function.[§]

The average urinary recovery of mannitol for all 92 periods was 100.4 mg for every 100 mg injected (range 62 to 156). The standard deviation of this series is 16.7; standard error of the mean 1.74. For the difference 100.4-100.0 the t value is 0.23, indicating that the amount recovered in the urine does not differ significantly from that injected.

In many of the clearances satisfactory mannitol plasma equilibrium was not achieved due to inaccurate preclearance estimation of renal function, a priming dose of improper size, changing renal function, etc. In addition, some periods are inaccurate due to technical difficulties such as obvious urine collection errors, chemical analytical errors, pump

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[†]National Institute of Health Senior Research Fellow and formerly Research Fellow in Pharmacology.

[‡]Harrison Fellow in Surgical Research, and formerly Research Fellow in Pharmacology.

¹Berger, E. Y., Farber, S. J., and Earle, D. P., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 62.

²Dominguez, R., Coreoran, A. C., and Page, I. H., *J. Lab. and Clin. Med.*, 1947, **32**, 1192.

³Elkinton, J. R., *J. Clin. Invest.*, 1947, **26**, 1088.

⁴Earle, D. P., and Berliner, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 262.

⁵Newman, E. V., Bordley, J., and Winternitz, J., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 253.

⁶Barker, H. G., and Clark, J. K., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 120.

⁷Smith, W. W., Finkelstein, N., and Smith, H. W., *J. Biol. Chem.*, 1940, **135**, 231.

⁸Coreoran, A. C., and Page, I. H., *J. Biol. Chem.*, 1947, **170**, 165.

⁹Smith, H. W. et al., *J. Clin. Invest.*, 1945, **24**, 388.

[§]Mimeographed sheets containing data will be furnished on request.

TABLE II.

Nocturnal Gastric Secretion: Average Volume, Free Acidity, and Output Free Hydrochloric Acid.

	Vol. (cc)	Free acid (Cl units)	Mg free HCl
Normal	581	29	661
Duodenal ulcer (series No. 1)*	1004	61	2242
Active uncomplicated duodenal ulcer (present series)	1047	58	2208
Healed uncomplicated duodenal ulcer (present series)	1002	54	1957

* For complete data see reference No. 2.

seemed desirable therefore to measure the nocturnal gastric secretion in a group of patients with uncomplicated active duodenal ulcer having distress, and to repeat the same determinations in the same individuals during a period when the ulcer was healed and symptoms were not present.

Methods. The 12-hour nocturnal gastric secretion was measured in 13 patients with duodenal ulcer during a period when their ulcer was easily demonstrable roentgenologically and when typical distress was present. The studies were repeated in the same individuals after medical treatment had led to healing of the ulcer roentgenologically and to the complete subsidence of symptoms; the latter studies were carried out 2½ months to 2¾ years after the ulcer was known to have healed.

The methods of study were identical with those described previously,^{1,2,3} enabling a strict comparison with our earlier observations. All data were submitted to statistical analysis. Previous studies have shown² that in the same individual with duodenal ulcer the total volume of the nocturnal gastric secretion on the average varies by approximately 20% and the free acidity and output of free hydrochloric acid by approximately 25%. This spontaneous variation was taken into account in evaluating the present results.

Results. The data for total volume, free acidity and output of free hydrochloric acid in the 12-hour nocturnal gastric secretion in the same individual during the phase of active ulcer and again during the period of healed ulcer are presented in Table I. The volume was unchanged in 9, significantly greater in 3, and significantly diminished in 1 (Case 6).

The free acidity of the total volume was unchanged in 11, and diminished in 2 (Cases 1 and 6). The output of free hydrochloric acid was unchanged in 9, significantly greater in 3, and diminished in 1 (Case 6). For the entire group there were no significant differences in the average volume, free acidity and output of free hydrochloric acid before and after healing of the ulcer (Table II).

The present data, in agreement with that obtained in previous studies^{1,2,3} (Table II), indicate that the average nocturnal gastric secretion in patients with uncomplicated duodenal ulcer and also in patients with healed uncomplicated duodenal ulcer is significantly greater than that of normal healthy individuals. Furthermore, the average night secretion of patients with healed uncomplicated duodenal ulcer is not significantly different from that of patients with duodenal ulcer requiring hospitalization because of distress (Series I).^{1,3}

The hourly pattern of acid secretion after the ulcer had healed was the same as had been noted during the period of active ulcer. The secretion of acid in all individuals in this series was continuous and was maintained at a relatively higher level than is seen in normal individuals. In no instance were there periods of anacidity for as long as one hour.

The persistence of hypersecretion in the vast majority of cases after healing of duodenal ulcer emphasizes the importance of continued careful antacid therapy in such patients.

Conclusions. 1. In the same patient with duodenal ulcer the 12-hour nocturnal gastric secretion is usually unaltered with healing of the ulcer.

2. The average 12-hour nocturnal gastric secretion of patients with active ulcer is not

³ Levin, E., Kirsner, J. B., Palmer, W. L., and Butler, C., *Gastroenterology*, 1948, 10, 939.

NOCTURNAL GASTRIC SECRETION AND DUODENAL ULCER

TABLE I.
Twelve-Hour Continuous Nocturnal Gastric Secretion in Patients with Duodenal Ulcer Before and After Healing.

Case	Uncomplicated active duodenal ulcer				Uncomplicated healed duodenal ulcer				% change
	Vol. (cc)	Free acid (Cl units)	Free HCl (mg)	Time interval (mo.)	Vol. (cc)	Free acid (Cl units)	Free HCl (mg)	Vol.	
1	1104	53	2130	6½	857	22	689	0	-40
2	737	51	1348		979	39	1375		
3	581	49	1035	4½	770	42	1163	+44	-
4	1466	52	933	3	778	35	991		+
5	1102	103	5481		1324	82	3954	0	-
6	718	115	4606	3	1128	94	3869		-
7	1398	13	333	3	818	18	536	+	0
8	1098	57	2887	3	720	20	524		+
9	643	67	2657		1430	60	3123	0	0
10	614	55	1290	3	1358	75	3707		+
11	675	52	1162		349	25	320	-52	-44
12	630	62	1523	30	258	34	317		+
13	840	40	917		1019	47	1726	+53	-74
14	1800	40	1223	9	1164	74	3122		+
15	1700	65	4259		1623	64	3787	0	0
16	1650	55	3403						
17	1600	62	3724						
18	1560	48	2795						
19	940	57	3237						
20	1396	112	3828						
21	1262	97	4921	1½ yr	1176	92	3933	0	0
22	1200	70	3211		1108	75	3044		0
23	1400	58	2533						
24	1500	55	2803	2½ yr	1344	56	2726	0	0
25	2000	61	3330		1394	63	3033		0
26	543	34	2476						
27	621	42	830	2 yr	731	47	1251		+
28	525	50	1130		654	53	1262	+	+
29	650	34	650	2½ yr	1039	16	618		0
30	500	20	473		897	23	767	+74	0
31	939	6	109	7					
32		45	1553		1109	37	1515	+	0
33					1032	42	1584		0

+ Slight but not significant increase.
Slight but not significant decrease.

TABLE I.
Some Effects of Triple Parabiosis in Rats—Joined from Days 25 to 50 and from Days 40 to 50.

	Days Joined	No. of Animals	Body wt (g)	Avg organ wt in mg per 100 g body wt		
				Ovaries	Uterus	Thymus
Center female	25-50	8	96	257	333	76
(attached to castrated male)	40-50	11	108	203	264	63
Right female	25-50	8	101	11	200	266
(attached to female)	40-50	11	113	27	170	241
Female controls		6	123	16	95	298
(littermates of parabionts)		8	146	29	152	296

Discussion. In triple parabiotic rats of which the left member is a castrated male, the known ability of gonadotrophic hormones to cross the parabiotic union^{3,4,5} is demonstrated by the stimulation of the ovaries of the middle female. The apparent lack of stimulation of the ovaries in the second female suggests that the gonadotrophins are utilized completely by the ovaries of the first female.

The reason for the increase in the weight of the uterus of the second female, in the absence of any enlargement of the ovaries, is not clear. Either there is some transfer of estrogen between the 2 females or the ovaries of the outer female, though reduced in size, are actually elaborating sex hormone. If estrogen from the center female is stimulating the uterus of the other female, a very high concentration of the hormone is indicated since it has been demonstrated that at least

40 times the minimal effective dose must be injected into one member of a pair of ovariectomized females which have been united in parabiosis in order to detect uterine stimulation in the twin.⁵ If the small ovaries of the outer female are stimulating its uterus then size of the ovary fails to measure physiological activity.

The slight decrease in the weight of the thymus of the second female is correlated with a proportionate increase in the weight of the uterus and is probably also due to the result of estrogens since gonadotrophins have no direct effect upon the thymus.^{6,7}

Summary. Gonadotrophic hormones from a castrated male cross successfully the first parabiotic union to stimulate the ovaries of the middle female of a set of triple parabiont rats. But the gonadotrophins are so completely utilized by the center animal that they produce no detectable ovarian stimulation of the second female.

³ Kallas, H., *Compt. Rend. Soc. Biol.*, 1929, **102**, 280.

⁴ Hill, R. T., *J. Exp. Zool.*, 1932, **63**, 203.

⁵ Biddulph, Clyde, Meyer, Roland K., and Gumbreck, Laurence G., *J. Exp. Zool.*, 1941, **88**, 17.

⁶ Evans, H. M., and Simpson, M. E., *Anat. Rec.*, 1934, **60**, 423.

⁷ Plagge, James C., *J. Morph.*, 1941, **68**, 519.

significantly different than in patients with healed duodenal ulcer.

3. Twelve-hour nocturnal gastric secretion in patients with healed duodenal ulcer and

who are without symptoms is significantly greater than that of normal healthy individuals.

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Can Gonadotrophic Hormones Cross Two Parabiotic Unions in Rats?

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It has been shown that when 3 rats are joined in parabiosis gonadotrophic hormones produced by the middle member of the triplet set affect both of the other rats at the same time.¹ The present experiment was designed to test the ability of gonadotrophic hormones to pass from one lateral member, through the middle animal, and into the opposite lateral triplet of 3 united rats. Preliminary results, reported in abstract form,² have been extended and confirmed.

Methods and Results. Results are given for 19 sets of triple parabionts. Eight sets were united on the 25th day of life and 11 on the 40th day. A total of 14 single females, littermates of the parabionts, were used as controls in the two groups. All animals were autopsied at the age of 50 days. The results for the 2 age groups substantiate each other.

In producing triple parabiosis the edges of the abdominal walls were sutured together without establishing a coelio-anastomosis. The scapulae were held together by 3 interrupted sutures. The skin was joined by metal wound clips from ear to base of tail after removing strips of skin from the adjacent sides of each animal.

Two females from the same litter were joined in parabiosis, and a castrated male was attached to the left sister to make the triplet set. In all but 2 cases the male was a littermate of the 2 females in the set. The passage of gonadotrophic hormones from the castrated

male to the center female was indicated by the enlarged ovaries and uterus and by the involuted thymus (Fig. 1 and Table I). The female to the right, not directly attached to the male, showed no ovarian stimulation, the ovaries weighing even less than those of the littermate controls. The uterus, however, appeared to be slightly stimulated and the thymus very slightly reduced in size. The stimulated ovaries of the middle female contained large follicles and large corpora lutea.

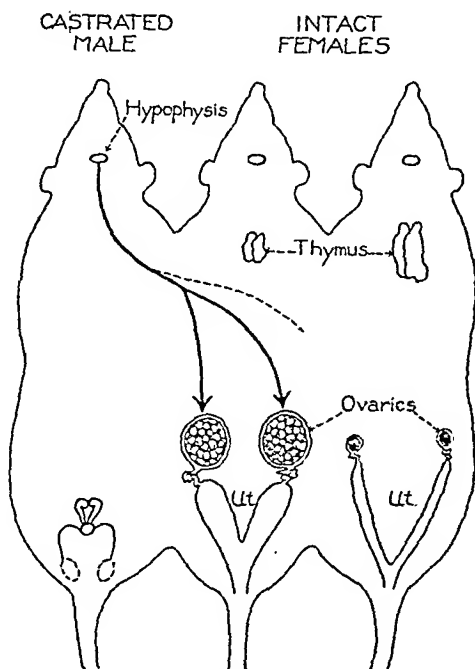


FIG. 1.

¹ Greep, R. O., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 214.

² Plagge, James C., *Anat. Rec.*, 1946, **94**, 489.

TABLE I.

Effect of Testosterone Propionate on the Body Weight, Penis Weight and Length of Os Penis in Rats Castrated and Hypophysectomized on Day 26 and Injected for 21 Days.

No. of rats	Daily dose test prop. mg	Age in days	Avg. wt gain g	Avg. wt distal penis, mg	Avg. length os penis, mm
3†		21			2.73 ± 0.11
3†		26			3.33 ± 0.15
7		47	9	23.5 ± 1.8 (5)*	3.20 ± 0.18 (6)
5	.01	47	5.6	57.3 ± 4.5	3.52 ± 0.14 (4)
8	.1	47	21.2	102.3 ± 4.3 (5)	3.80 ± 0.12
7†		47	103.6	81.5 ± 19.3 (5)	4.1 ± 0.2 (5)

± = Standard deviation.

* Figures in brackets indicate the number of rats (when less than the total) used in a calculation, the penes of those excluded being used for different purposes.

† Normal unoperated controls.

planes in a way that permitted one to obtain representative sections through the greatest length and the central part of the os. Measurements of the bone were made with a calibrated oculo-micrometer. Enough of the patellae were sectioned and stained to provide the information that this cartilage, showing only the earliest signs of ossification on day 26, changes in the 3 weeks after hypophysectomy and gonadectomy even in the untreated rats to a well ossified structure somewhat smaller than in the 47-day-old normal animals.

Results. Table I summarizes the results. It was known from previous experiments carried out in this laboratory that hypophysectomized rats of this age might be expected to gain slightly in weight. The average weight increments of 9 g in 21 days for the operated controls and 5.6 g for the rats on the 0.01 mg level of testosterone propionate are not considered unusual, but the 21.2 g gain with a range from 15-32 g in the 8 rats receiving the higher dose of androgen must be viewed as an effect of the hormone not mediated through the pituitary. Although this gain in weight (1 g per day) parallels that observed in hypophysectomized rats given low dosage of pituitary somatotrophin, it must be considered small (20%) when contrasted with the weight increment in normal control rats of this age. However the penes of the operated rats injected with the 0.1 mg level of testosterone propionate had developed at a slightly faster rate than those of the normal animals; and the average weight of their distal segments exceeded that of the normal rats by 25%. The os penis of the

androgen-treated rats, then, may be considered a bone within an organ growing even more rapidly than in the normal animals. The higher dose of androgen permitted the os penis to attain a length approximately 19% greater than the uninjected, operated controls and 92% as great as normal rats of a similar age. The lower dose was less effective as may be seen in Table I. The main histologic findings are represented in the photomicrographs and briefly summarized in the legends.

Discussion. Adequate discussion of these findings must be postponed until further experiments are carried out to determine the possible intermediary role played by organs other than the pituitary (e.g., the thyroid). The growth autonomy of certain parts of the skeletal system as shown by tissue culture experiments⁴ and as re-emphasized by the Dutch investigators who witnessed bone growth in rats hypophysectomized on day 5⁵ has a place in final interpretations.

The present findings support the earlier evidence that testosterone propionate may cause skeletal growth changes¹ and body weight increase^{6,7} in rats in the absence of the pituitary. This in no way detracts from the importance of the pituitary's role in promoting growth. Rather, it suggests that along with its so-called direct growth hormone

⁴ Fell, H. B., and Robinson, R., *Biochem. J.*, 1929, **23**, 767.

⁵ Van Eck, W. F., and Freud, J., *Acta brev. Neerl.*, 1941, **11**, 43.

⁶ Leonard, S. L., *Endocrinol.*, 1943, **32**, 229.

⁷ Gordon, G. S., Evans, H. M., and Simpson, M. E., *Endocrinol.*, 1947, **40**, 375.

Androgen-Induced Growth of the Os Penis of Hypophysectomized-Gonadectomized Rats.*

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It was concluded from earlier experiments carried out by other investigators in this laboratory¹ that under certain circumstances testosterone propionate promoted growth of cartilage and bone in the proximal tibial epiphysis of hypophysectomized rats. In the report of that work it was suggested that explanations for apparent discrepancies noted in the literature dealing with the effects of androgens on bone growth may be found in such variables as age, sex, dosage, length of treatment, etc. In the experiment outlined below a new variable has been introduced in that the bone chosen for study, the os penis, is peculiarly situated in a region that might be expected to be affected profoundly by the deprivation or varying supply of androgenic substances. Using normal and castrated rats with intact pituitaries Turner *et al.*² showed that testosterone propionate accelerated the development of this bone as judged by skiagrams.

Experimental. It was previously determined³ that rats of the Long-Evans strain gonadectomized on day 26 and injected daily thereafter for approximately 3 weeks with 0.01 mg of testosterone propionate would show growth of the penis and splitting of the balano-preputial epithelium comparable to that of the normal animal. This dose was therefore assumed to approximate the daily average equivalent of androgen to which the normal animal was exposed during that period of life. As may be seen, then, from Table I some rats hypophysectomized and gonad-

ectomized on day 26 were immediately started on this daily dose of testosterone propionate[†] while others received 0.1 mg. Control animals also doubly-operated on day 26 received 0.1 cc of sesame oil, the volume of diluent used for the hormone.

Terminal normal controls were obtained from the same litters as the experimental rats and kept on the same regime of diet and care. Control tissues from 21- and 26-day-old normal rats as well as from full grown animals were also studied. In this preliminary experiment the rats were allowed to eat *ad lib.*, but it is planned to control the important variable of food-intake in a future experiment in this series. Animals were weighed on days 5, 10, 15, and 21 of the experiment and it was obvious from the appearance of the food cups and the weight gains that the operated animals ate well, although never as much as the normal controls. On the other hand, the normal rats were more active and possessed better body tone.

At necropsy the penis trimmed of skin, and a patella from each animal were fixed in Bouin's fluid, and the operative sites were checked for completeness. After 2 days in Bouin's fluid the os penis becomes decalcified as judged by skiagram, but the tissues were also exposed to 5 parts of concentrated HCl (by volume) to 95 parts of the graded alcohols (50-100%) over another 2-day period. At the 80% alcohol stage the penis was uniformly cut in such a way that the final block of tissue consisted of the distal segment and only the bend of the proximal segment. After the excess fluid was removed from these blocks of tissues they were weighed (see Table I). The penis was cut in dorso-ventral

* Aided by grants from the Research Board of the University of California.

¹ Simpson, M. E., Marx, W., Becks, H., and Evans, H. M., *Endocrinol.*, 1944, **35**, 309.

² Turner, H. H., Laehmann, E., and Hellbaum, A. A., *Endocrinol.*, 1941, **29**, 425.

³ Lyons, W. R., Berlin, I., and Friedlander, S., *Endocrinol.*, 1942, **31**, 659.

[†] Generously supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

FIG. 1. The os penis of a rat castrated and hypophysectomized on day 26 and injected, subcutaneously, daily, thereafter for 21 days with 0.1 mg testosterone propionate in sesame oil. Necropsy one day after the last injection. Condyles have developed proximally. The completely ossified distal tip is in close contact with the urethra of the glans and is inserted against a cushion of dense connective tissue and fibro-cartilage which later forms a secondary ossicle. The marrow cavity is inconspicuous except in the condylar region. The balano-preputial epithelial cord has split under the influence of the androgen to form a horny, papillated covering for the glans (G) and the smooth lining of the prepuce (P). See Fig. 6 for higher magnification of the proximal end of the os. H. & E. $\times 25$.

FIGS. 2-7. All H. & E. $\times 90$. Proximal end os penis.

FIG. 2. From an onset control rat 26 days old. Chondrocytes are being formed from the chondrogenic tissue (above) and are being destroyed and replaced by trabeculae of endochondral bone. Periosteal bone is also being formed here and was observed throughout the remaining length of the os. The marrow contained but few fat cells.

FIG. 3. From a 9-month-old, unoperated, untreated, normal rat. Apparently the definitive pattern of this bone has been attained. Although no cartilage cells were recognized the possibility that some of the mesenchyme covering the bone might become chondrogenic remains. The marrow is almost entirely fatty.

FIG. 4. From a 47-day-old rat, hypophysectomized and castrated on day 26 and injected only with sesame oil as a control. Cartilage has disappeared and osteoblasts line communications between the marrow cavity and what was formerly perichondrium (above). Most of the marrow is composed of fat cells. Some of the rats in this group showed a few remaining cartilage cells in this region of the os.

FIG. 5. From a 47-day-old rat, hypophysectomized and castrated on day 26 and injected daily immediately thereafter with 0.01 mg of testosterone propionate. Cartilage has been maintained and has continued to proliferate and to be replaced by bone.

FIG. 6. Same, except for hormone dosage which was 0.1 mg. The marrow in Figs. 5 and 6 has been restored to a condition similar to that of a normal rat of this age (see Fig. 7).

FIG. 7. From a normal, untreated, 47-day-old rat.

(somatotrophin) effect and its indirect action upon skeletal tissues through thyrotrophin, one must also consider the indirect stimulus of its gonadotrophic substances responsible for the elaboration of androgen. Both male hormone and somatotrophin have been shown to perform nitrogen sparing (protein-anabolic) functions^{7,8} and Rupp and Paschkis have recently reminded us that thyroid hormone at optimal levels is also a protein anabolizer.⁹

Summary. Rats hypophysectomized and castrated on day 26 and injected immediately thereafter with 0.1 mg testosterone propionate

daily for 21 days showed an average daily weight gain of 1 g. During the same period unoperated, uninjected littermate controls gained approximately 5 g daily. The distal segment of the penis of these experimental animals weighed on an average 25% more than the normal 47-day-old controls. The ossicle in this portion of the penis of the experimental group measured 3.80 ± 0.12 mm as contrasted with the normal length of 4.1 ± 0.2 mm and the original length of 3.33 ± 0.15 mm for the 26-day-old normal and the length of 3.20 ± 0.18 mm for the doubly-operated 47-day-old controls uninjected with hormone.

Addendum. Our attention has been called to similar findings reported by Sindram, I. S., *et al.* in *Acta Neel. Morph.*, 1939, 2, 236.

⁸ Koehakian, C. D., Conference on Metabolic Aspects of Convalescence, N. Y., 1946, p. 169.

⁹ Rupp, J., and Paschkis, K. E., Abstract 58, 1948 Meeting of the Assoc. for the Study of Internal Secretions.

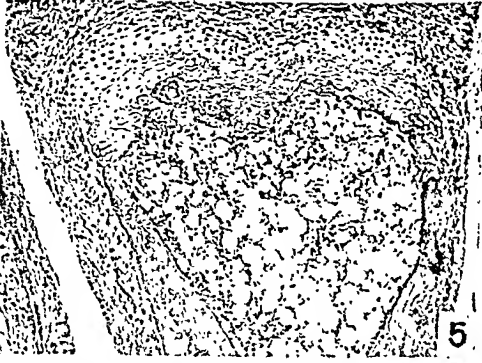
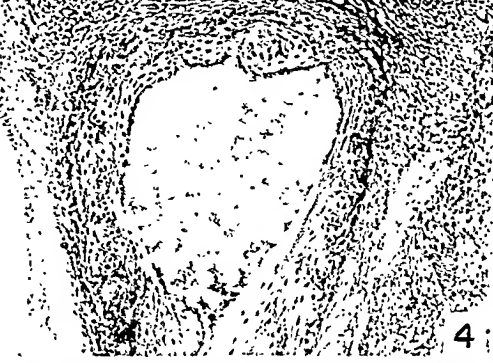
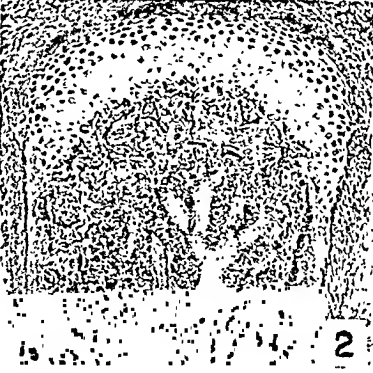
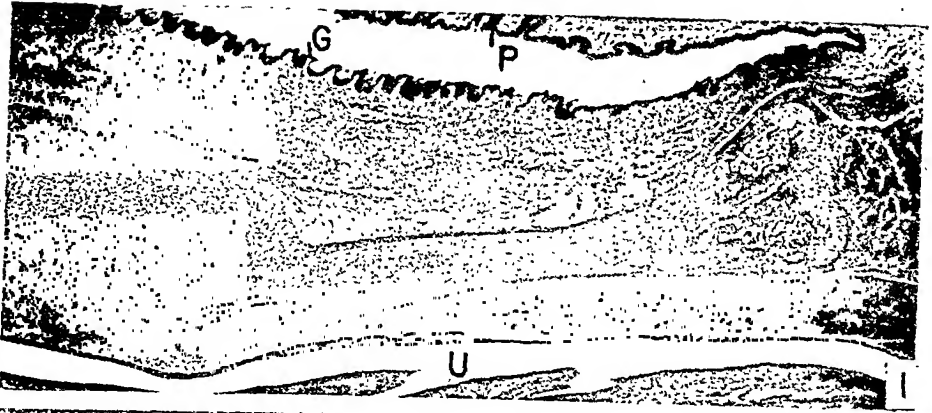


TABLE I.
Activity of C¹⁴ in Tissue Proteins in Counts per mg per Minute.*

Organ	Tryptophan deficiency		Phenylalanine deficiency			
	C ¹⁴ -Tryptophan administered		C ¹⁴ -Tryptophan administered		C ¹⁴ -Glycine administered	
	Supplemented	Deficient	Supplemented	Deficient	Supplemented	Deficient
Intestines	64.8	28.9	43.9	38.8	197	174
Plasma	52.0	28.0				
Liver	41.5	27.9	20.3	17.7	140	105
Kidney	34.9	21.4	29.7	20.6	118	68.0
Spleen	22.7	9.6	28.2	17.5	114	90.2
Testes	19.6	5.4				
Stomach	30.3	8.0				
Heart	10.5	4.3	8.4	5.1	43.0	29.4
Brain	10.2	4.3				
Skeletal muscle	4.7	2.7	4.5	1.8	23.4	5.0
Red cells	5.0	2.0				

* The data represents the average value of 2 animals in each group.

deficiency both when the animals are starved or fed. The decrease may be due to a decrease in net synthesis of protein or to a lowered rate of turnover, or to both combined. The deficiency of the amino acid could directly limit the quantity of enzymes responsible for the incorporation of the label or the effect

may be an adaptive response.

Summary. Animals reared on a diet deficient in an essential amino acid (tryptophan, phenylalanine) showed a reduced incorporation of tracer amounts of C¹⁴ from labeled amino acids in the tissue proteins.

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Passive Transfer of Local Cutaneous Hypersensitivity to Tuberculin.

M. N. METAXAS AND M. METAXAS-BÜHLER. (Introduced by Merrill W. Chase.)

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Many attempts have been made in the past to transfer tuberculin hypersensitivity passively to normal animals. Most of these attempts have been unsuccessful, and the positive results claimed by some authors have either been seriously contested by others, or have been too irregular to be quite convincing.^{1,2} A method which insures a readily reproducible transfer of tuberculin hypersensitivity was first described by Chase³; this

method had been previously devised by Landsteiner and Chase⁴ for the passive transfer of the "delayed type" of skin sensitivity to simple chemical compounds. Briefly, it consists in injecting a suspension of living exudate, spleen or lymph node cells from actively sensitized guinea pigs into the peritoneal cavity or the blood stream of normal animals, following which the latter develop general cutaneous hypersensitiveness to tuberculin within 1 to 3 days. Using the same or slightly modified methods, Kirchheimer and Weiser,⁵ Cummings, Hoyt, and Gottshall,⁶

¹ Zinsser, H., and Mueller, J. H., *J. Exp. Med.*, 1925, **41**, 159.

² Rich, A. R., *The Pathogenesis of Tuberculosis*, p. 398, 2nd printing, Springfield, Charles C. Thomas, 1946.

³ Chase, M. W., *Proc. Soc. Exp. Biol. and Med.*, 1945, **50**, 134.

⁴ Landsteiner, K., and Chase, M. W., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 688.

⁵ Kirchheimer, W. F., and Weiser, R. S., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 166.

Effect of Amino Acid Deficiencies on Incorporation of Radioactive Carbon-Labeled Amino Acids into Animal Tissue Proteins.*

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Comparatively little is known of the effect of malnutrition on the metabolic processes of the organism. Accumulation of such information is of importance for the advancement of biochemistry. In the present investigation it is shown by means of isotopic tracer experiments that deficiency of either of the essential amino acid tryptophan or phenylalanine results in impaired ability of the mammalian organism to incorporate C^{14} from labeled amino acids into protein.

The introduction of protein-hydrolysates in therapy has led to the development of the concept that all essential amino acids must be present simultaneously for protein synthesis and growth. The injection of tryptophan a few hours after the injection of a casein-hydrolysate containing no tryptophan failed to induce positive nitrogen balance, while simultaneous administration of the two was successful.¹ Melnick and coworkers² attributed the lower biological value of soy protein compared to that of heat-processed soy protein, to a delayed liberation of methionine during digestion in the gastrointestinal tract. When an incomplete mixture of amino acids was fed to rats and the missing essential amino acid supplemented after an interval of several hours, the animals did not grow.³ It was of interest, therefore, to study the behavior of a tracer dose of a labeled amino acid in an essential amino acid deficiency to obtain information on the effect of the malnutrition on the C^{14} uptake by the tissue pro-

teins of the organism.

Young male rats (Long Evans strain) were placed on a tryptophan deficient diet.⁴ Another litter-mate group received the same diet with 0.5% L-tryptophan added. After about 8 weeks, when the symptoms of deficiency had developed, DL-tryptophan- β - C^{14} (0.85 μ C per mg or 105,000 counts per minute per mg) was injected intraperitoneally (3 mg per 100 g body weight). The animals were then fasted for 8 hours and sacrificed. Protein samples from different tissues were prepared for radioactivity assay by homogenizing the tissues and precipitating and washing the protein with trichloroacetic acid followed by washings with hot alcohol, alcohol-ether mixture and ether. The radioactivity counts were corrected for self absorption from a curve prepared by counting a highly radioactive sample of protein in layers of different thickness.

In another series of experiments, phenylalanine deficiency was produced in male mice on a synthetic diet,⁵ the control group receiving in addition 1.2% DL-phenylalanine. After 3 to 4 weeks, one set of mice received intraperitoneally (3 mg per 100 g body weight) carboxyl-labeled glycine (5.37 μ C/mg) and another set received labeled tryptophan (2 mg/100 g). The animals were killed 8 hours after injection as in the previous experiment, but in this case they had free access to food. The proteins of the tissues were treated as before.

It is seen from the data (Table I) that the incorporation of the label into the tissue proteins is reduced in essential amino acid de-

* Aided by grants from the Rockefeller Foundation and the Research Funds of the Medical School.

† Indian Government scholar.

¹ Elman, R., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 484.

² Melnick, D., Oser, B. L., and Weiss, S., *Science*, 1946, **103**, 326.

³ Geiger, E., *J. Nutr.*, 1947, **34**, 97.

⁴ Berg, C. P., and Rose, W. C., *J. Biol. Chem.*, 1929, **82**, 479.

⁵ Synthesized by Dr. Charles Heidelberger and supplied through the generosity of Dr. Heidelberger and Dr. Melvin Calvin.

⁵ Womack, M., and Rose, W. C., *J. Biol. Chem.*, 1947, **171**, 37.

cc saline subcutaneously, and 15 minutes later 1.25 cc O.T. + 1.25 cc saline intraperitoneally. Most of the "Fernausslösung" experiments were carried out in this manner.

The results of both types of experiment were clear-cut. The reactions at the skin sites prepared with "tuberculous" cells were well developed 16 hours after tuberculin testing and usually at their height at 24 hours. At this time they presented the aspect of rather small but characteristic tuberculin reactions: an indurated, blanched or livid area (4-9 mm in diameter), surrounded by a diffuse zone of erythema significantly wider than before the injection of tuberculin (10-15 mm). In several instances the center was hemorrhagic, thus completing the picture to that of a typical "cocarde" reaction. Strongly reacting sites became necrotic after 48 to 72 hours, and sloughed off in the next few days. It should be noted that all the reactions were of the delayed type; occasionally very slight blanching of a sensitized site was seen as early as 7 hours after the O.T. injection, but never any earlier. In contrast, no change whatever was observed in the control sites after the introduction of tuberculin at a remote point, and, as has already been mentioned, only a slight increase in inflammatory symptoms was seen after local tuberculin testing.

By way of illustration in one experiment 4 young albino guinea pigs received 0.039 cc "tuberculous" cells intracutaneously in the right flank, and 0.042 cc "normal" cells in the left. The sequence of reactions in one of these animals was as follows:

Forty-eight hours after the cell injections: in both flanks areas of swelling and redness (6x6 mm) with a small yellowish center (1x1 mm). 0.25 cc O.T. + 0.25 cc saline are injected under the abdominal skin, followed 15 minutes later by 1.25 cc O.T. + 1.25 cc saline i.p. Immediate shock of moderate intensity; recovery complete in a few minutes. Twenty-four hours later the inflamed area in the left flank is unchanged. Reaction in the right flank: 8x9 mm white

indurated area, sharply demarcated, with 4x5 mm violet-red (hemorrhagic) center; outer zone 10x12 mm angry red diffuse margin. Forty-eight hours after tuberculin: 6x8 mm black necrotic center, 10x12 mm red outer zone; the former sloughed off in a few days.

Five experiments of the kind described above were performed, involving a total of 41 animals, 21 of which were used as cell recipients. In 15 of these, transfer of tuberculin hypersensitivity was successful, as evidenced by medium to strong tuberculin reactions at the specifically prepared skin sites. In one experiment an attempt was made to determine whether local sensitivity is preceded by a latent period: the 5 recipients used, including one animal tested 48 hours after the cell injections, gave negative or at best only suggestive reactions, and had to be recorded as failures. In another experiment the duration of local reactivity was explored; this was found to last up to 5 days, and the one recipient in the experiment which was tested 7 days after sensitization failed to react at all. Tests carried out with control glycerine broth gave entirely negative results, whether the broth was injected locally into prepared skin sites or intraperitoneally (when it also caused anaphylactoid shock).

Conclusions. The method of local passive sensitization so extensively employed in human allergy (Prausnitz-Küstner reaction) and in experimental anaphylaxis (Opie and others), can also be applied to the cellular transfer of tuberculin hypersensitivity discovered by Chase.³ Compared with the latter's experimental procedure, this method offers the advantage of considerably reducing the number of cell donors necessary for successful transfer. The technic used is, however, susceptible of improvement, as in the presence of a marked primary reaction of cavy skin to the injection of homologous exudate cells none but fairly strong tuberculin reactions can be recorded with confidence.

and Stavitsky⁷ have obtained similar results. The object of the present study was to determine whether passive tuberculin hypersensitivity can also be induced locally by the intracutaneous injection of exudate cells from tuberculin sensitive animals.*

The methods were based on those used by Chase,³ and adapted to the special purpose of this work outlined above: Guinea pigs weighing from 550 to 950 g were used as cell donors. They were infected with a recently isolated virulent human strain of *M. tuberculosis*, by the subcutaneous injection of 0.2 mg (moist weight) of bacilli in saline. 3½ to 7 weeks later the animals were skin-tested with O.T. before each experiment, and those showing the strongest reactions were selected for the production of peritoneal exudate. Each experiment comprised 2 tuberculous and 2 normal cell donors. All 4 animals received 30 cc of paraffin oil intraperitoneally. Forty-eight hours later they were killed, and the peritoneal cavities were washed out with heparinized Tyrode solution. The "tuberculous" and the "normal" exudate cells were recovered from the washings separately by mild centrifugation and washed three times in fresh Tyrode. Finally they were made up with heparinized Tyrode into very thick suspensions, similar in appearance and consistency to slightly diluted pus. 0.1 cc of "tuberculous" and of "normal cell" suspension was then injected intracutaneously into each flank of a young albino guinea pig, 3, 4 or 5 such cell recipients (between 250 and 400 g in weight) being used in each experiment. A few drops of the cell suspension left in the

injection syringe were filled into a short capillary tube, which was then centrifuged at 2,500 r.p.m. for ½ hour. In this tube the cell volume/suspension volume ratio could be accurately determined; it amounted to about 2/5, and as it remained constant on further sharp centrifugation, it was adopted as a reliable basis on which to calculate the cell volume actually injected. Thus each cutaneous bleb contained 0.035-0.045 cc of cells, the difference in volume between "tuberculous" and "normal" cells in any particular experiment never being more than 0.005 cc. The cells consisted of 57-75% large mononuclear cells, 8-36% lymphocytes, and 6-22% polymorphonuclear leucocytes, the "tuberculous" cells usually comprising more lymphocytes and less polymorphonuclears than the "normal" cells.

The injection of cell suspension elicited a marked reaction in the skin of the recipients; this consisted, 48 hours after the injection, in a moderately indurated circular area of erythema measuring between 5 and 9 mm in diameter, with a small yellowish center, rarely wider than 1 mm. At this time the recipients were tested with tuberculin. For this purpose two technics were employed: a) the direct injection into the prepared skin sites of 0.1 cc O.T. 1/10; and b) the injection at a distant point (intraperitoneally or subcutaneously) of 1.0-1.5 cc O.T., diluted in an equal volume of saline. The first method was not quite satisfactory, as the control sites (prepared with "normal" cells) reacted to the direct introduction of tuberculin with a slight increase in swelling and redness. This was entirely avoided with the second method ("Fernausslösung"), which was adopted for most of the experiments. The intraperitoneal injection of O.T., however, in the amounts mentioned above, resulted in every case in a fairly severe (but never lethal) anaphylactoid shock. On the other hand, the subcutaneous injection of 1.5 cc of undiluted O.T. was unsatisfactory, owing to protracted absorption of the depot, which remained visible through the abdominal skin for several days. It was felt that the severity of shock was somewhat reduced by the following combination of both routes of administration: 0.25 cc O.T. + 0.25

* Cummings, M. M., Hoyt, M., and Gottshall, R. Y., *Pub. Health Rep.*, 1947, **62**, 994.

⁷ Stavitsky, A. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 225.

* H. J. Corper and R. E. Stoner have also attempted to induce passive local hypersensitiveness to tuberculin in guinea pigs by the intracutaneous injection of a mixture of tuberculin and citrated whole blood from actively sensitized animals (*Amer. Rev. Tub.*, 1946, **54**, 305). Their uniformly negative results were probably due to the low proportion of white cells in whole blood, the relatively high dilution of tuberculin used, and the simultaneous injection of both reagents.

the piece of ileum which was subjected to frequent stimulation began to show marked and reproducible changes in its reaction to histamine, it was replaced by a control strip. Then, by registering the contractions, it was observed whether the control strip, which had not been stimulated before, showed the same types of response.

Results. In the guinea pig ileum, in non-atropinized media, the contractions caused by acetylcholine and by histamine mutually reinforced each other during the first hour after death. (Fig. 1, 2). Toward the end of the third hour when histamine was added to the bath, after the intestine had been contracted by acetylcholine, there was a small initial contraction immediately followed by a pro-

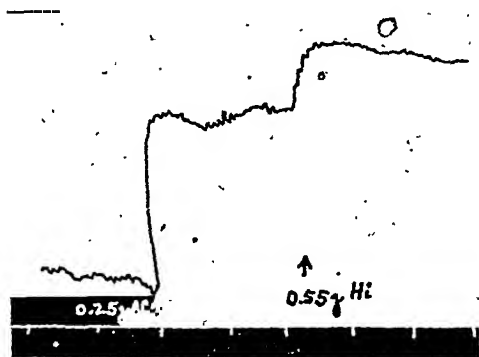


FIG. 1.

Effect of acetylcholine and histamine on the isolated guinea pig ileum $\frac{1}{2}$ hour after death of the animal. Consecutive contractions are additive. Time in 30 second intervals.

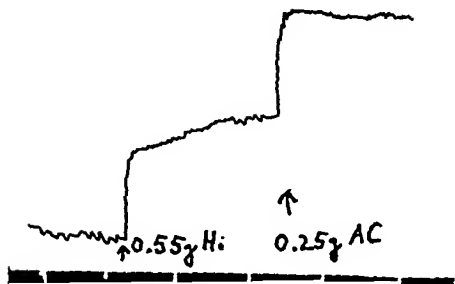


FIG. 2.

Effect of histamine and acetylcholine on the isolated guinea pig ileum $\frac{1}{2}$ hour after death of the animal. Time in 30 second intervals.

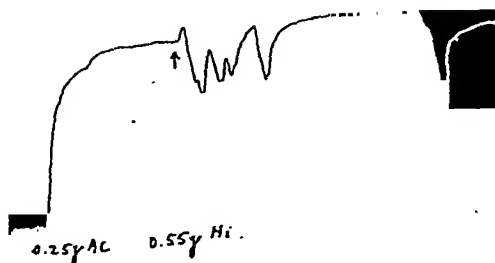


FIG. 3.

Effect of acetylcholine and histamine on the isolated guinea pig ileum $3\frac{1}{2}$ hours after death of the animal. Histamine causes relaxation when added after acetylcholine.

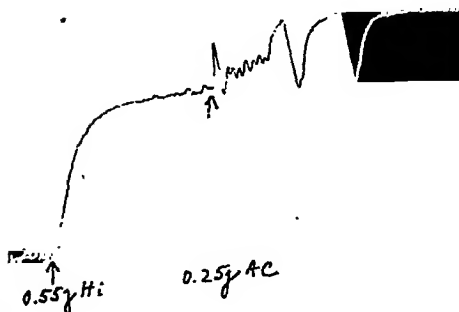


FIG. 4.

Effect of histamine and acetylcholine on the isolated guinea pig ileum $3\frac{1}{2}$ hours after death of the animal. Acetylcholine reinforces the contraction caused by histamine.

found relaxation. The muscle then recovered and gradually reached the level of contraction attained prior to the addition of histamine. (Fig. 3.) The situation was different when, at the height of a histamine contraction, acetylcholine was added to the bath. Acetylcholine always reinforced the histamine contraction. At the beginning of the experiment simple summation occurred. However, toward the end of the third hour, the contraction initiated by the addition of acetylcholine was interrupted by a series of large peristaltic waves. These in-

Action of Acetylcholine, Histamine and Cholinesterase Inhibitors on Isolated Ileum in Relation to its Survival Time.*

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In preliminary experiments it was found that the atropinized ileum of the guinea pig began to react to acetylcholine $2\frac{1}{2}$ hours after the death of the animal. Observations of this nature were made by Le Heux,¹ who showed that the rhythmic peristaltic contractions of the isolated guinea pig ileum could no longer be blocked by atropine after the intestine had been kept in Locke solution for several hours; he also noticed a similar behavior in the isolated small intestine of the rabbit. We therefore decided to study the correlation between the time elapsed after death and the responses to acetylcholine, histamine and various cholinesterase inhibitors of the atropinized and non-atropinized intestinal strips of several species of animals.

Experimental. Guinea pigs, rabbits, rats and chickens were used. Strips of ileum were suspended in a Tyrode bath of 45 ml at 37°C. The temperature of the bath for the chicken intestine was kept at 41°C. The experiments were started within 15 minutes after death.

At the beginning of each experiment, several successive doses of 5 to 10 μ g acetylcholine bromide were added to the bath. At the height of the second contraction caused by acetylcholine (one minute after addition of the drug), histamine phosphate was added to the bath. After each contraction, the Tyrode solution was changed and a resting period of 3 minutes was allowed.

Acetylcholine bromide and histamine phosphate were added in amounts which caused submaximal contractions. Satisfactory results were obtained by adding the following doses, expressed in μ g, to a bath of 45 ml volume:

	Guinea pig	Rat	Rabbit	Chicken
Acetylcholine bromide	0.2-0.5	0.2-0.6	0.2-0.6	0.05-0.3
Histamine phosphate	0.5-1.0	60.-150	10.-40	5.-10

These doses correspond to those recommended by other authors.²⁻⁶

The cholinesterase inhibitors were used in the following amounts: Physostigmine sulfate: 0.5-10 μ g; neostigmine methylsulfate: 1-10 μ g; hexaethyl tetraphosphate: 10 μ g.

When the intestine was treated with both acetylcholine and histamine, the second drug was added only after the first contraction had reached its maximum. The same precaution was observed when histamine was followed by acetylcholine.

In one series of experiments the intestinal strips were suspended in atropinized Tyrode solution. The intestine was considered to be completely atropinized when it failed to respond to repeated additions of 10-50 μ g acetylcholine bromide. For this purpose the following concentrations of atropine were found to be satisfactory: 1 mg atropine sulfate per liter Tyrode for the guinea pig, 2 mg per liter for the rat and 3 mg per liter for the chicken and rabbit. Histamine was used in the same amounts as in the case of non-atropinized intestines.

In all experiments control strips from the same animal were kept in regular and atropinized Tyrode solution for several hours at room temperature or in a bath at 37°C. When

² Levy, J., and Michel, Z., *J. Physiol. et Pharm. gén.*, 1937, **35**, 389.

³ Kahlson, G., *Arch. f. exp. Path. u. Pharmacol.*, 1934, **175**, 189.

⁴ Page, I. H., and Schmide, E., *Z. f. Physiol. Chem.*, 1930, **191**, 262.

⁵ Ambache, N., *J. Physiol.*, 1946, **104**, 266.

⁶ Ersparmer, V., *Arch. f. exp. Path. u. Pharmacol.*, 1940, **106**, 343.

* This work was aided by a grant from the Office of Naval Research N6ori-20, Task Order 11.

¹ Le Heux, J. W., *Arch. f. d. ges. Physiol.*, 1920, **170**, 177.

choline and to histamine. A decreased sensitivity of the isolated guinea pig ileum to histamine in the springtime has been reported by Kwiatkowski.⁷

In all experiments, the atropinized ileum of all 4 species did not react to acetylcholine and to the cholinesterase inhibitors during the first 2 to 3 hours after death. At the time when the non-atropinized intestine began to fail to contract from histamine, the responses of the atropinized muscles to acetylcholine and to cholinesterase inhibitors were no longer blocked by atropine in the same concentration which was sufficient to inhibit the action of acetylcholine immediately after death. A typical experiment is shown in Table I.

Discussion. From our experiments it seems that several hours after death a certain change occurs in the intestinal muscle. This change in the surviving tissue results in a reversal of the response to histamine at a time when the muscle still contracts to acetylcholine and to the cholinesterase inhibitors. These observations do not support the recent theory⁵ that histamine contracts the smooth muscle by causing a liberation of acetylcholine. The different character of the contractions caused by histamine and by the cholinesterase inhibitors and the different reactions to atropine are also against the theory that histamine stimulates the smooth muscle of the intestine through a cholinergic mechanism. On the other hand, the possibility cannot be

excluded that there is "an interdependence between the sites of attack" of histamine and acetylcholine as was suggested by Rocha e Silva.⁸ It is also obvious that when experiments are carried out with isolated intestinal muscle, it is necessary to take into account the time which has elapsed since the death of the animal, particularly when histaminic responses and the reactions of the atropinized muscles to parasympathetic stimulants are being observed.

Summary. The action of acetylcholine, histamine, physostigmine, neostigmine, and hexaethyltetraphosphate was studied on the isolated ileum of the guinea pig, rabbit, rat and chicken in non-atropinized and atropinized media. In all 4 species the contractions caused by acetylcholine and histamine reinforced each other immediately after the death of the animal, regardless of the order in which they were added to the bath. Two and a half hours to 3 hours after death, histamine failed to contract the intestine when it was added at the height of an acetylcholine contraction. In the rat, rabbit and chicken the intestine no longer responded to histamine at a time when it still reacted to acetylcholine and to the cholinesterase inhibitors. In all 4 species atropine failed to block the action of acetylcholine and cholinesterase inhibitors in intestinal strips which were at least 3 hours old.

⁷ Kwiatkowski, H., *J. Physiol.*, 1943, **102**, 32.

⁸ Rocha e Silva, M., *J. Pharm. Exp. Ther.*, 1944, **80**, 399.

TABLE I.
Sensitivity of the Isolated Guinea Pig Ileum to Acetylcholine at Different Intervals After Death.

Time after death in min.	Acetylcholine to 45 ml bath fluid μ g	Height of contraction in mm
10	10	42
15	10	72
20	10	96
25	10	96
30	10	57
At 35 min. atropinized Tyrode was added (1 mg atropine sulfate/liter)		
40	10	0
90	10	0
120	10	0
135	10	0
140	10	3
150	10	9
180	10	18
195	10	15

creased in magnitude and frequency as the experiment proceeded. Eventually the muscle reached a plateau which was invariably higher than the one which it maintained after the first addition of histamine. (Fig. 4) Control muscles kept in Tyrode solution for several hours before the addition of histamine reacted in the same way as those which had undergone frequent stimulation.

In the rabbit ileum histamine and acetylcholine mutually reinforced each other during the first 2 hours after death. From the third or fourth hour on, a summation occurred only when histamine was followed by acetylcholine, but not when acetylcholine was followed by histamine.

During the first hour after death histamine elicited contraction of the rat intestine. As early as 2 hours after death the same dose of histamine caused relaxation or provoked no response. In the chicken intestine, 3 hours after death, the reaction to histamine became reversed at a time when the intestine was still responding with the usual contraction to the addition of acetylcholine.

The contractions caused by the cholinesterase inhibitors had the following characteristics: 1. The response was delayed while the response to histamine was always immediate. The delay between the addition of the drug and the contraction was most marked in the guinea pig. In all 4 species the time interval

between the addition of the drug and the contraction was longest with physostigmine, least pronounced with neostigmine. 2. The response was gradual, *i.e.*, it took several minutes before the muscle reached the height of its contraction. Within the range of doses used, the delay in the response and the amplitude of the contraction were largely independent of the dose administered. 3. The intestinal strips still reacted to acetylcholine and to the cholinesterase inhibitors with a contraction at a time when histamine provoked no more response or relaxation only.

A total of 12 experiments was carried out on rabbits, 20 on rats and 9 on chickens. Of these 9 rabbits, 18 rats and 6 chickens showed the above described phenomena. Without exception, the small intestines of the individuals which did not undergo these changes responded with unusually sluggish contractions both to acetylcholine and to histamine. In some instances such delayed responses could be ascribed to the fact that the animals were old, or that the intestines had been subjected to prolonged handling before stimulation. However, in the case of the guinea pig, in all 24 animals which were used during the winter months, the reaction of the intestine underwent the changes described above. In 8 experiments which were carried out in the spring on the same strain of guinea pigs, the intestine reacted in a sluggish way to acetyl-

TABLE I.

Renal Plasma Flow in cc per Minute, Corrected to 1.73 sq. m. Body Surface, During and After Work of Relatively Long Duration at the Rate of 3 m.h.p. at 5% Grade.

Subject	Basal	Work					Recovery		
		20 min.	40 min.	1 hr	2 hr	3 hr	20 min.	40 min.	60 min.
BS	512	416	315	332	360	355	452	458	471
PW	576	499	487	459	425	425	—	—	—
WW	523	484	431	452	434	462	—	557	723
SN	607	466	385	402	345	399	456	566	535
Mean	555	466	405	411	391	410	454	516	578

work of this type, the increase in pulse rate and oxygen uptake, for example, are maximal 10 to 15 minutes after initiation of exercise.

Presumably, as the subject approaches exhaustion other changes in the renal plasma flow take place but in none of our experiments were the subjects seriously taxed by the work imposed.

Recovery was studied in 3 subjects for one hour after cessation of exercise. Although the number of experiments is too small to permit statistical testing, the speed of the recovery process after 3 hours of exercise seems to be much the same as after only 32 minutes of this work. In neither case was it as rapid as return of the pulse rate or blood pressure to resting levels.

Summary. 1. Renal plasma flow was studied by the para-amino-hippurate clearance method during moderate exercise of relatively long duration in 4 healthy young men. 2. During the first 16 minutes of walking 3 m.p.h. at 5% grade the renal plasma flow fell from 9 to 23.2% (of the resting control); during the second 16 minute period it fell from 15.4 to 36.6% and during the third 16 minute period from 18.5 to 33.7%. 3. During the second and third hours of exercise (2 consecutive 56-minute periods), there was little further change in the renal plasma flow. 4. In 2 of 3 experiments where recovery was studied, the renal plasma flow had returned to within 10% of the resting control value one hour after cessation of exercise.

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Asymmetrical Suppression of Vertebral Epiphyseal Growth with Ionizing Radiation.

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(Introduced by L. J. Soffer.)

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Ionizing radiation can suppress growth of epiphyses. Many studies of this phenomenon have been made on long bones, but only Engel¹ has previously described the deformities resulting from irradiation of the growing spine. He placed radium needles alongside the spines of young goats and produced a wedge deformity of the adjacent vertebrae.

The narrowing of the vertebrae occurred on the side nearer the radium, and the resultant scoliosis was concave on the same side.

We have tried additional technics in radiation of the spine in growing animals. Using radon seeds for interstitial radiation and 140 kV x-rays for external radiation, our preliminary experiments have attempted to establish a useful dosage range for suppression of vertebral epiphyseal growth in baby rabbits.

* Dazian Research Fellow in Radiology.

¹ Engel, D., *Am. J. Roentgenol. and Rad. Therapy*, 1939, 42, 217.

Renal Plasma Flow During Moderate Exercise of Several Hours' Duration in Normal Male Subjects.

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That exercise produces a decline in renal plasma flow is now established.^{1,2} In a previous report² the degree of reduction was found to be progressive for at least 30 minutes as exercise proceeds and was directly related to the severity of the exercise. The effects of long-sustained exercise have not, to our knowledge, been determined and it is with this topic that the present report deals.

Method. Technically the method employed in this work was identical with that previously described² except that the time spent on the treadmill totalled 3 hours. Para-amino-hippurate was used to determine renal plasma flow. As in the previous experiments a moderate water diuresis was instituted and urine samples were collected by voluntary micturition. The exercise was at the rate of 3 miles per hour at 5% grade.

Material. Four normal male students, aged 22 to 27 years, were employed for the study. Three of the subjects had been studied extensively in previous experiments on the effects of exercise of relatively short

duration. A single experiment employing exercise of long duration was done on each subject.

Results. Fig. 1 and Table I summarize the effects of long-continued exercise on the renal plasma flow in these 4 subjects. At this level of exercise the renal plasma flow declined to a relatively stable value during the first 40 to 60 minutes of exercise. The value reached during the first hour was relatively well maintained in these 4 subjects for the remaining 2 hours of exercise. The degree of decline in the first 32 minutes of work corresponded almost exactly to that determined in previous experiments² at the same level of work.

In 2 of the subjects the renal plasma flow had returned to within 10 per cent of resting levels 40 minutes after cessation of work. In a third subject the resting value was slightly exceeded at the end of 40 minutes. No studies of recovery were carried out on the fourth subject.

Discussion. The decrease in renal plasma flow in response to moderate exercise is, it appears, a progressive phenomenon only during the first 40 to 60 minutes of the exercise. Continuation of the stress for 2 full hours beyond this point resulted in no further decline in the amount of plasma flowing through the kidney. It is well established that the cardiovascular system can adjust relatively quickly to various levels of work, permitting the work to be continued without further circulatory adjustment until the subject approaches the point of exhaustion. That the kidney participates in the circulatory changes is clearly shown in this and previous experiments. The shunting of blood away from the kidney reaches a maximum relatively early in sustained exercise and is continued until the work ceases. The adjustment of renal plasma flow in response to exercise is, however, not as rapid as some other circulatory changes. In

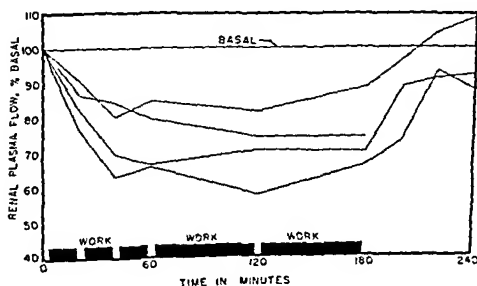


FIG. 1.

Changes in renal plasma flow in 4 normal male subjects during and after walking 3 m.p.h. at 5% grade.

¹ Barclay, J., Cooke, W., Kennedy, R., and Nutt, M., *J. Physiol.*, 1945-46, 104, 14P.

² Chapman, C., Henschel, A., Minckler, J., Forsgren, A., and Keys, A., *J. Clin Invest.*, 1948, 27, 639.

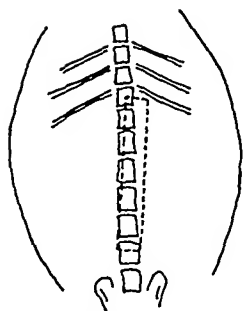


FIG. 3. (Tracing from roentgenogram).

This shows the spine of a three week old rabbit with 1×7 cm radiation field indicated by dotted line. Dose to the middle of the vertebral bodies involved was 1000r.

gastro-intestinal infections. The five survivors received dosages to the region midway between the anterior and posterior surfaces of the vertebral bodies of 100r, 350r, 700r, or 1000r. Only the 2 animals which received the highest dosage showed marked wedging on the irradiated side of the vertebrae. (Fig. 4). Slight wedging was seen in an animal which received 700r.

Summary. The lumbar spines of 30 young rabbits were unilaterally irradiated with radon seeds and X-rays. Twenty-one animals died of gastro-intestinal disturbances unrelated to radiation. Five of the 9 survivors showed wedging of the vertebral bodies with the narrowing of the bodies on

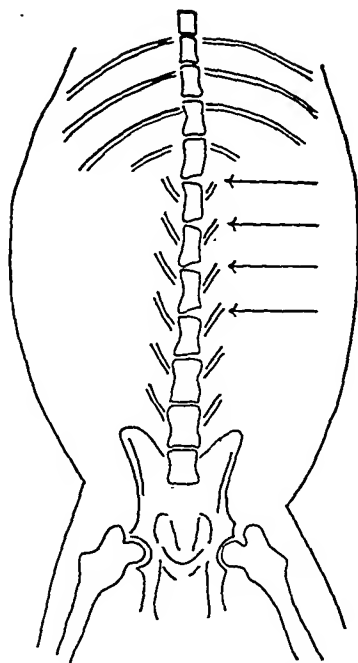


FIG. 4. (Tracing from roentgenogram).

This shows the spine of the same rabbit 54 days later. Wedged vertebrae are indicated by arrows.

the more heavily irradiated side. Dosage estimates indicate that growth of the epiphyses of the vertebral bodies of baby rabbits may be suppressed with 700 to 1000 r to the epiphysis. Doses of 350 r or less have no apparent effect on the growth.

Interstitial Radiation. Radon seeds were implanted just lateral to a lumbar intervertebral space. Films of the spine were made with seeds *in situ* (Fig. 1) in order to evaluate positioning and dosage. Fourteen rabbits had a total of 24 seeds implanted; 10 rabbits had two seeds in each, and 4 animals had 1 seed in each. These seeds were equivalent to 0.2, 0.4, or 0.6 millicurie of radium on total decay and were filtered by the equivalent of 0.3 mm of platinum. Of these 14 animals 10 died of gastro-intestinal infections less than 3 weeks after implantation of radon seeds. The 4 surviving animals had a total of 6 seeds. Wedging occurred adjacent to 2 seeds, both of which were placed in apposition with the intervertebral space and contained 0.6 mc of radon on total decay. (Fig. 2) Other implanted sites did not show wedging because of poor positioning of seeds or lower radon content of the seeds.

External Radiation. Asymmetrical radiation of the growing epiphyses of the vertebral bodies was also effected by directing a narrow beam of x-rays to one side of the lumbar spine. Under intravenous anesthesia to immobilize the rabbits one side of the spine was treated with dosages (in air) ranging from 300 to 1200r. Radiation factors were: 140 kV, 20 ma., 30 cm target skin distance, filtration of 0.25 mm Cu, and 1 mm Al., HVL 0.50

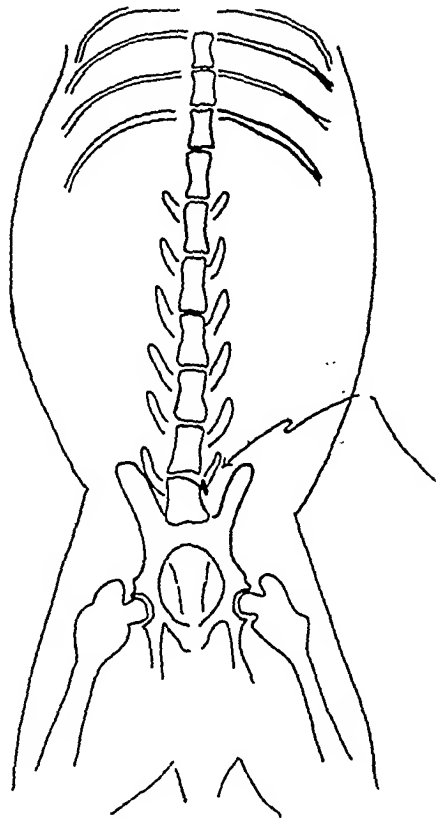


Fig. 2. (Tracing from roentgenogram).

Same rabbit as in Fig. 1. This film was made 110 days after implantation of radon seed and illustrates asymmetrical growth of both vertebrae adjacent to the 0.6 mc. radon seed (arrow). In this instance the middle of the epiphyses nearest the seed received an estimated dose of 2680r while the opposite side of these vertebral epiphyses received 670r. The growth suppression varied directly with the dosage.

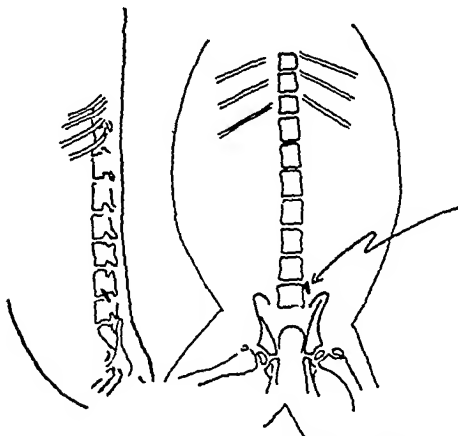


Fig. 1. (Tracing from roentgenogram). Anteroposterior and lateral roentgenograms made immediately after implantation to show position of implanted radon seed (arrow). The spine is straight.

mm Cu., and a flux of 60 r/min. The field size was 1 x 7 cm, and the dose at the middle† of the vertebral bodies at a depth of 1.5 cm was 70% of the air dose^{2,3} at the center of the field. Accuracy of field was verified by exposing x-ray films with the rabbits under anesthesia and with the therapy machine in place ready for treatment (Fig. 3).

Of rabbits irradiated externally 11 died of

† Midway between anterior and posterior edges.

² Glasser, O., Quimby, E. H., Taylor, L. S., and Weatherwax, J. L., *Physical Foundations of Radiology*, Paul B. Hoeber, Inc., N. Y., 1944.

³ Clarkson, J. R., *Brit. J. Radiol.*, 1941, 14, 265.

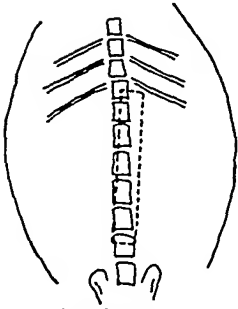


FIG. 3. (Tracing from roentgenogram).

This shows the spine of a three week old rabbit with 1×7 cm radiation field indicated by dotted line. Dose to the middle of the vertebral bodies involved was 1000r.

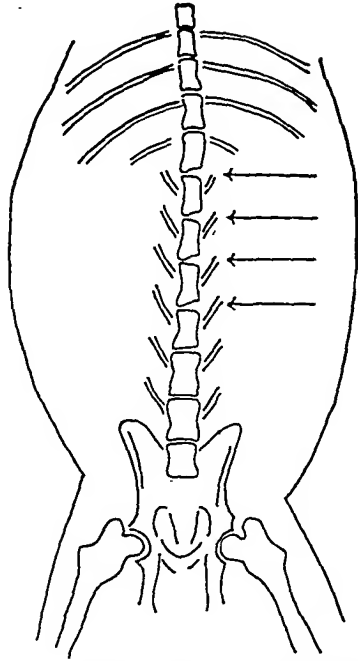


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Summary. The lumbar spines of 30 young rabbits were unilaterally irradiated with radon seeds and X-rays. Twenty-one animals died of gastro-intestinal disturbances unrelated to radiation. Five of the 9 survivors showed wedging of the vertebral bodies with the narrowing of the bodies on

the more heavily irradiated side. Dosage estimates indicate that growth of the epiphyses of the vertebral bodies of baby rabbits may be suppressed with 700 to 1000 r to the epiphysis. Doses of 350 r or less have no apparent effect on the growth.

Urinary Excretion of Aureomycin.*

H. S. COLLINS, E. B. WELLS,[†] T. F. PAINE, JR.,[‡] AND M. FINLAND.[§]

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Aureomycin is a new antibiotic which is active against many gram-positive and gram-negative bacteria and also against certain viral and rickettsial infections.⁶ As part of a clinical trial of this antibiotic in a number of infections, bacteriologic and pharmacologic studies were also carried out in this laboratory. The results of the bacteriological studies are reported elsewhere.¹ In this paper are presented some preliminary findings on the urinary excretion of aureomycin after single and multiple doses.

Two normal adult male subjects were given a single dose by mouth in the morning on a fasting stomach; one was given 0.5 g and the other 0.75 g. Each subject emptied his bladder just prior to taking the dose and the urine thus voided was used as a control. The bladder was then emptied at intervals, the volumes of urine recorded and aliquots immediately filtered,** frozen and stored at -20°C . After all the urines had been collected, each specimen was assayed for aureomycin by a method similar to that used by Rammelkamp for

penicillin.² The test organism, *Streptococcus* No. 98, was completely inhibited by 0.5 μg per ml and partially inhibited, as evidenced by growth on subculture, by 0.25 to 0.125 μg per ml. The control urine specimens, obtained before and several days after the dose, failed to inhibit the growth of the test organism.

The results for the subject receiving 500 mg are shown in Fig. 1 and those for the subject receiving 750, who had a much greater volume of urine output, are shown in Fig. 2. The greatest rate of aureomycin excretion occurred in both instances between 4 and 8 hours. If similar absorption and excretion take place from repeated oral doses, the intervals between such doses should be about 8 hours in order to maintain maximum absorption and excretion rates. Concentrations of aureomycin sufficient to completely inhibit the test organisms when the urine was diluted 16-fold or more were excreted for at least 33 hours. The aureomycin was still being ex-

* Aided by a grant from the United States Public Health Service.

[†] Research Fellow in the Field of Virus Diseases, National Research Council.

[‡] Research Fellow, American College of Physicians.

[§] Clare Wilcox, Janice M. Bryan, and Paul F. Frank rendered technical assistance.

[¶] Reports on the method of isolation of aureomycin, its pharmacology and activity will be made by workers of the Lederle Laboratories Division of the American Cyanamid Company at the New York Academy of Sciences, July 21, 1948. Some of the data were made available by these workers in personal communications to the authors.

¹ Paine, T. F., Jr., Collins, H. S., and Finland, M., *J. Bact.*, 1948, 50. Submitted for publication.

** Filtration through Seitz, Mandler or sintered glass filters did not remove any of the antibiotic.

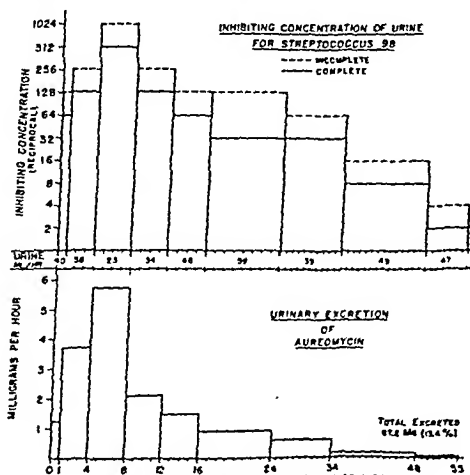


FIG. 1.

² Rammelkamp, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1942, 51, 95.

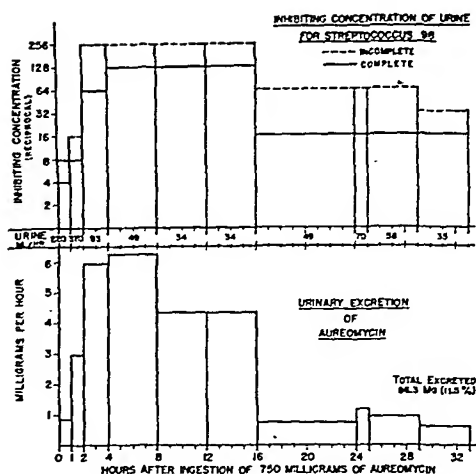


FIG. 2.

creted in both subjects at 55 and 33 hours, respectively, when the studies were terminated. The total amount recovered was 13.4% of the 500 mg dose in 55 hours and 11.5% of the 750 mg dose in 33 hours.

Blood levels carried out with the same organism were unsatisfactory since the maximum amount of antibiotic in the serum inhibited the test organism only in a final dilution of 1:2 or 1:4.

Total urinary excretion studies were made in 2 patients who were receiving 2 doses of aureomycin by mouth each day for several days. The urines were collected at 12-hour intervals and all voidings were stored at 5 to 10°C during the intervals. The tests were otherwise carried out in the same manner as

before. In one of these patients who received 0.25 g twice a day for 10 days the concentrations of antibiotic in the urine were sufficient completely to inhibit the test streptococcus in dilutions of 1:4 to 1:16, and partial inhibition occurred in dilutions of 1:8 to 1:32. In the patient who received 0.5 g twice a day for 7 days the concentrations of aureomycin in the urine ranged from 10 to 32 μ g per ml. In these 2 patients the total amount recovered was 5% and 2%, respectively of the amount administered. Antibiotic activity was present in the urine up to 72 hours and longer after the last dose. Control urines from these patients, obtained before and several days after the antibiotic was given failed to inhibit the test strain.

Both of the patients were acutely ill and the differences in the percentage recoveries may have been related to poor absorption of the drug associated with the illness. Technical difficulties may also have accounted, in part, for the discrepancies.

Summary. After a single oral dose of 0.5 or 0.75 g of aureomycin given to fasting normal subjects antibiotic activity was recovered in the urine for more than 33 to 55 hours. The antibiotic was excreted in high concentrations between 2 and 16 hours and the largest rate of excretion occurred between 2 and 8 hours. The findings suggest that the optimum intervals between oral doses of aureomycin should be about 8 hours.

16658 P

Stereoencephalotomy.*

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Using the previously described stereotaxic apparatus (stereoencephalotome) for the production of subcortical lesions in the human

brain,¹ the following procedures have been developed.

I. Dorsomedial thalamotomy in mental dis-

* Aided by a grant from the Am. Med. Assn. Committee on Scientific Research.

¹ Spiegel, E. A., Wycis, H. T., Marks, M., and Lee, A. J., *Science*, 1947, 100, 349.

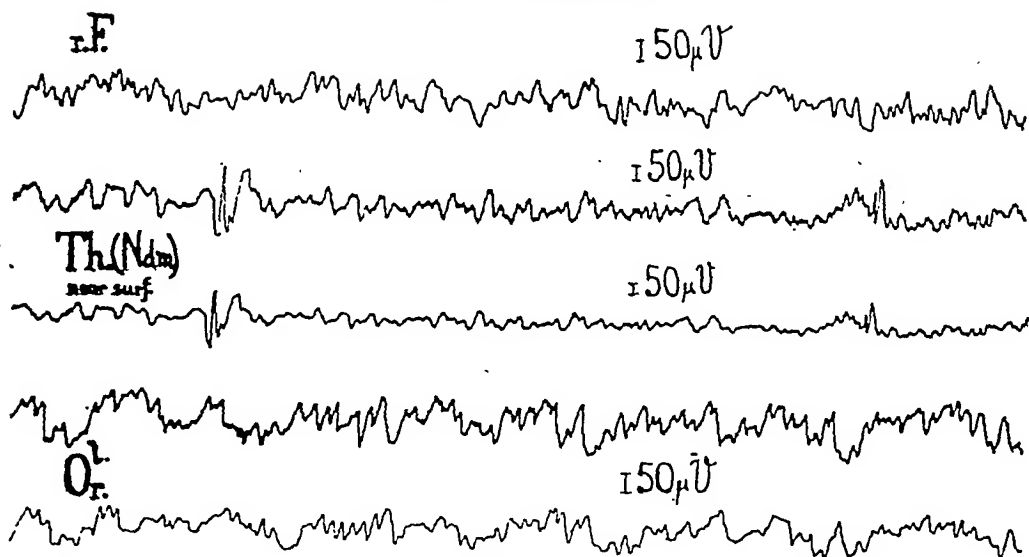


FIG. 1.

Electrothalamogram (Th) recorded from the dorsomedial nucleus near its surface simultaneously with the electroencephalogram recorded from the scalp in the frontal (F) and occipital (O) areas in a patient suffering from grand and petit mal attacks (patient under pentothal-ether-nitrous oxide anesthesia).

orders. Lesions have been placed bilaterally in the dorsomedial nucleus of the thalamus in mental disorders with a prevalent emotional component, in order to interrupt the cortico-diencephalic connections probably related to the mechanism of emotions (16 cases). Lesions covering about one seventh of the volume of this nucleus on each side have been able to produce relief of nervous tension, anxiety, depression, irritability, agitation, hallucinations or compulsions; this has been obtained² without at least some of the undesirable by-effects of prefrontal lobotomy such as epileptiform convulsions, childishness, facetiousness, distractibility, lack of tact or discipline.

II. *Mesencephalothalamotomy* (mty) for relief of intractable pain. Lesion of the long ascending pain-conducting pathways in the midbrain has been combined with a lesion of the dorsomedial nucleus. Since patients become unconcerned about their pain following prefrontal lobotomy (Freeman and

Watts³), and since the dorsomedial nucleus degenerates following this procedure, a lesion of this nucleus is added to that of the long ascending pain-conducting pathways in order to induce a relative indifference to pain perceived through remaining auxiliary pathways. In a woman suffering from unilateral facial pain for 6 years despite retrogasserian rhizotomy, mty on the opposite side abolished the pain completely except for occasional paresthesias in the maxillary region.⁴ In a second patient suffering from diffuse burning pain and spasms in the right lower extremity following an injury to the lumbar spine and unrelieved by sympathectomy, rhizotomy and bilateral chordotomies, the bilateral mty reduced the painful area to the region around the right buttock, while the spasms of the leg and the sensation of the muscular contractions persisted.

III. *Paracommissural thalamotomy* in petit mal. In cases suffering from grand and petit mal attacks, lesions were placed bilaterally at the level of the commissura media

² Spiegel, E. A., Wycis, H. T., and Freed, H., Joint Meeting Philadelphia and New York Neurology. Soc., April 23, 1948.

³ Freeman, W., and Watts, J. W., *Lancet*, 1946, 1, 953.

⁴ Spiegel, E. A., and Wycis, H. T., *Wien. med. Wochenschr.*, in press; Wycis, H. T., Soloff, L., and Spiegel, E. A., *Philadelphia Neurolog. Soc.*, Feb. 27, 1948.

around the medial part of the internal lamina medullaris.⁵ Electric stimulation of this region in experimental animals yields a spike and dome pattern in the electroencephalogram (Jasper⁶). Preceding this lesion, the electrothalamogram was recorded simultaneously with the electroencephalogram;⁷ spike discharges were found in the electrothalamogram.[†] These spike discharges were not

⁵ Spiegel, E. A., Wycis, H. T., Freed, H., and Lee, A. J., *Am. Neurol. Assn.*, June 16, 1948.

⁶ Jasper, H. H., and Droogelever-Fortuyn, J., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1947, **26**, 272.

⁷ Wycis, H. T., Spiegel, E. A., and Lee, A. J., *Intl. League against Epilepsy, Am. Branch*, June 13, 1948.

[†] Gibbs, Hayne and Gibbs⁸ also recorded spike discharges from various subcortical areas in petit mal. The authors inserted the recording electrode into the brain of the patients without the guidance of a stereotaxic apparatus, trying to establish the location by x-ray studies after air injections into the ventricles.

limited to the immediate vicinity of the internal lamina medullaris but sometimes showed the maximum in more dorsal parts of the thalamus close to the ventricle (Fig. 1). In two cases, the operation did not influence the grand mal attacks; it diminished, at least transitorily, the frequency of the petit mal attacks and the severity of accompanying motor phenomena.

Summary. The stereotaxic technique has been applied to the human brain for relief of mental disorders with a prevalent emotional component and of intractable pain. In epileptics suffering from grand and petit mal, the electrothalamogram was recorded, revealing spike discharges; lesions placed in the vicinity of the commissura media diminished frequency and severity of the petit mal attacks.

⁸ Gibbs, F. A., Hayne, R., and Gibbs, E. L., *Intl. League against Epilepsy, Am. Branch*, June 13, 1948.

16659

Possible Role of Free Phenols in Renal Uremia.*

JOHN C. HARTNETT. (Introduced by H. B. Pierce.)

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The work of Becher and his associates,¹⁻⁶ has stressed the importance of free phenols in blood as a major factor in the development and intensification of the uremic syndrome.

* Data from thesis submitted in partial fulfillment of the requirements for the Degree Master of Science.

¹ Becher, E., *Deut. Arch. f. Klin. Med.*, 1925, **145**, 333.

² Becher, E., *Ergeb. d. ges. Med.*, 1933, **18**, 51.

³ Becher, E., and Koch, F., *Deut. Arch. f. Klin. Med.*, 1923, **148**, 78.

⁴ Becher, E., and Litzner, S., *Klin. Wochschr.*, 1926, **5**, 147.

⁵ Becher, E., and Litzner, S., *Klin. Wochschr.*, 1926, **5**, 1373. Cited *Chem. Ab.*, 1926, **20**, 3192.

⁶ Becher, E., Litzner, S., and Taglich, W., *Z. f. Klin. Med.*, 1926, **104**, 182.

In an extensive review of the pathogenesis of this syndrome, Harrison and Mason⁷ state that "whether in patients with nephritis phenol retention is sufficiently marked to produce further renal damage is an interesting but still unsettled problem." Dickes,⁸ using the method of Theis and Benedict⁹ for determining blood phenols, noted that 21 out of 23 uremic patients showed a direct correlation between the degree of cerebral depression and the height of the blood phenols. However, the correlation of total phenols was

⁷ Harrison, T. R., and Mason, M., *Medicine*, 1937, **10**, 1.

⁸ Dickes, R., *Arch. Int. Med.*, 1942, **21**, 662.

⁹ Theis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1924, **61**, 67.

TABLE I.
Blood Phenol Determinations—Method of Schmidt.
Diagnosis: Uremia of Renal Origin.

Case No.	N.P.N. mg %	Free phenol mg %	Conjugated phenol mg %	Total phenol (Free plus conjugated) mg %
1	105	.050	.519	.569
2	210	.164	—	—
3	—	.098	1.477	1.575
4	50	.077	.526	.603
5	59	.074	—	—
6	—	.077	.371	.448
7	92	.081	.145	.226
8	50	.099	—	—
9	51	.580	.229	.809
10	88	.089	.846	.935
11	189	.102	.878	.980
12	78	.051	.052	.103
13	54	.135	.425	.560
14	55	.073	.110	.183

better than that of the free or conjugated phenols. Roen,¹⁰ using the Millon reagent, reported an elevation of blood phenols in all cases of uremia regardless of the type of renal failure. Although the height of the blood phenols was generally related to the uremic syndrome, it was not in direct proportion to the uremic state.

A review of the literature concerning the methods for determining blood and urine phenols by Deichmann and Schafer¹¹ and Volterra¹² showed that there were very few, if any, methods available that were specific, or nearly so, for determination of the phenols in blood and urine. The methods used by Dickes⁸ and Roen¹⁰ and earlier by Becher *et al.*,¹³ because of the technic used or the non-specificity of the colorimetric reagent, gave phenol values which were high for human blood, if compared with values obtained by Deichmann and Schafer¹¹ or with those obtained by the method of Schmidt¹⁴ which eliminates most non-phenolic substances that

would affect the final colorimetric reading.

The method of Schmidt¹⁴ for determining blood phenols was critically evaluated by the author. Phenol recovery experiments were run on both beef and human blood. Average recovery of free phenol from beef blood was 77% and from human blood, 68%. Determinations were made on 14 normal human blood samples to obtain a point of reference in evaluating data obtained from bloods of uremic patients. Free and conjugated phenols in blood samples from patients diagnosed as having or developing the uremic syndrome from renal disfunction were determined. The results for free, conjugated and total phenols and those for non-protein nitrogen are shown in Table I.

Each clinical case, except No. 9, shows the free phenol value to be within a normal range (.051 to .088 mg %) as determined on the above mentioned series of normal adult blood. (Schmidt¹⁴ presented "normal" free phenol values for pooled human blood ranging between .011 and .041 mg %). Case No. 9 died 8 hours after the blood sample was taken for analysis. At this late stage of the syndrome the conjugating mechanism was undoubtedly failing rapidly, possibly accounting for the increased free phenol value.[†] It is important to note that, according to the

[†] P.M. findings: Liver: Chronic passive congestion. Kidney: Degenerated and resembling bichloride of mercury poisoning.

¹⁰ Roen, P. R., *J. Urol.*, 1944, **51**, 110.

¹¹ Deichmann, W. B., and Schafer, L. J., *Am. J. Clin. Path.*, 1942, **12**, 129.

¹² Volterra, M., *Am. J. Clin. Path.*, 1942, **12**, 525.

¹³ Becher, E., Litzner, S., and Taglich, W., *Munchen. Med. Wochschr.*, 1925, **76**, 1676.

¹⁴ Schmidt, E. G., *J. Biol. Chem.*, 1943, **150**, 69.

¹⁵ Nesbit, R. M., Burk, L. B., and Olsen, N. S., *Arch. Surg.*, 1946, **53**, 483.

free phenol values, not at any time is there present enough free phenol in the blood to produce the symptomatic picture of phenol poisoning or the uremic syndrome. This is contrary to the evidence of Becher¹ and Becher and Koch.³ The present author, with Becher and Litzner,⁴ Becher *et al.*,¹³ Dickes,⁸ Roen¹⁰ and Nesbit *et al.*,¹⁵ finds that the conjugated and hence the total phenols increase in the uremic syndrome, although the values given in this report are much lower than those of these authors.

Summary. In 14 clinical cases involving

uremic syndrome herein reported upon, it was found that:

a) The free phenols had little if any effect in producing or intensifying the uremic syndrome. b) During the uremic syndrome the free, conjugated and total phenols did increase, but not significantly enough to warrant their consideration as an important factor in the uremic syndrome. The methods employed in previous reports give reason to doubt the validity of conclusions drawn concerning the relationship of free, conjugated and total phenols to the uremic syndrome.

16660

Fluorimetric and Biological Determination of Estrogens in the Eggs of the American Lobster (*Homarus americanus*).^{*†}

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The presence of hormones in the tissues and body humors of a variety of invertebrates, both marine and terrestrial, appears to be well-established.¹ Among these, substances having estrogenic potencies are not uncommon.² However, satisfactory procedures for producing extracts free of contamination and for testing the potency of such extracts by fluorimetric, as well as the usual biological technics, have only recently become available. This study was made to determine the estrogenic potencies of the eggs of a representative marine invertebrate—the American lobster.

Materials and Methods. A total of 395 g

(wet weight), about a quarter of a million eggs, were used, the total yield of 9 specimens. All the eggs were recently spawned and attached to the caudal appendage. The eggs of the lobster remain within the body cavity for nearly a year following ovulation so that the eggs used in this study were all about 11 months old. The extraction procedure follows: The eggs are thoroughly macerated in a Waring Blendor in twice their volume of 95% ethyl alcohol and allowed to stand for 24 hours. The alcohol is filtered off and the residue re-extracted with alcohol for 24 hours. The alcoholic extracts are combined and shaken thoroughly for a half hour with an equal volume of petroleum ether. The ether fraction is removed and the alcoholic fraction is again shaken with ether. Following this, the alcoholic fractions are combined and evaporated to dryness and the deep orange residue is taken up in toluene as a supersaturated solution. For purposes of further purification preceding fluorimetric and biological analysis 5 cc samples were taken of the toluene concentrate. Five cc of the toluene solution are shaken with 10 cc 1N NaOH

* The cost of this investigation was defrayed by a grant from the Carnegie Foundation Fund for the Advancement of Teaching.

† The author is indebted to Dr. Robert W. Bates, Endocrine Products Division, E. R. Squibb & Sons, for his guidance in working out the chemical procedure and for his invaluable aid in the fluorimetric studies. The laboratory assistance of E. J. Collins is gratefully acknowledged.

¹ Hanström, B., *Hormones in Invertebrates*. Oxford Press, 1939, Chap. 2, p. 22.

² Donahue, J. K., *Endocrinology*, 1940, **27**, 149.

and slowly centrifuged for 10 minutes. The oily top layer is carefully removed and kept. The NaOH layer is neutralized to pH 3 with concentrated HCl under a small quantity of toluene. The toluene fraction is removed and kept. The oily fraction from the first NaOH separation is treated 5 times in the manner just described and the toluene fractions combined and reduced in volume to about 5 cc. Where the toluene samples appear slightly cloudy owing to the presence of small amounts of oil, another extraction with NaOH may be required.

Fluorimetric Studies. Colorimetric methods for the quantitative determination of estrogens have been greatly improved³ but where relatively minute amounts of estrogen are involved, fluorimetric analysis appears to be more sensitive.⁴⁻⁶ For purely qualitative analysis 0.2 to 0.5 cc of the purified toluene extract were placed in a fluorimetric tube and heated with 1 cc 90% sulfuric acid in a water bath at 80°C for 10 minutes. When held up to a narrow beam of light and viewed at an angle of 90° extracts prepared as described showed a blue-green fluorescence easily discernible with the naked eye.

For quantitative work the procedure outlined by Bates and Cohen was followed.⁵ Using a Coleman photofluorometer fitted with a B₁ filter at the photocell, subtracting the non-specific fluorescence developed by a blank solution, and comparing the intensity of fluorescence given by the sample with that developed by a standard solution of estrone, it was estimated that each cc of toluene contained between 30 and 50 international units of activity. However, later determinations made in Dr. Bates' laboratory employing a filter combination consisting of a 420 mμ Baird Interference Filter at the light source and a combination of a 520 mμ Baird Interference Filter and a 3387 Corning Filter at the

photocell gave readings of the order of 5 units per cc of toluene. It is believed that this filter combination eliminates virtually all non-specific fluorescence. On the basis of these readings it is estimated that the original 395 grams of eggs yielded between 400 and 500 units of recoverable activity.

Biological Studies. The response of the 150 g spayed rat to injections or vaginal applications of corn oil extracts of the toluene solutions correlated quite closely to the activity as judged by fluorimetric analysis. Ten 150 g rats were injected subcutaneously with the equivalent of 2.5 cc of toluene solution in 3 divided doses over a period of 3 days. The activity was transferred to corn oil by extracting the toluene solution with NaOH and then neutralizing the NaOH to pH 3 under a small quantity of ethyl ether which was mixed with corn oil and allowed to evaporate at room temperature. Ten more spayed females were given direct applications of corn oil solutions to the vagina in the same dosage as above. In taking smears and making vaginal applications great care was taken to avoid trauma. Within the 3-day period all animals showed a clearing of leucocytes and the appearance of large numbers of nucleated epithelial cells. In the injected series 2 animals showed some cornified cells at the end of 3 days. In the intravaginal group 5 of the 10 animals showed large numbers of cornified cells although in no cases were there full estrus responses. Each animal received the equivalent of 1.2 μg of estrone so that, in view of the observation that the rat is some 10 times less sensitive to estrone than the mouse, the dosage given would be close to the threshold necessary for a full response. Until larger quantities of eggs are obtained and highly concentrated extracts prepared from them the consistent production of full estrus smears must wait. In all animals tested, histological study of the reproductive tracts showed varying degrees of growth of the vaginal epithelium up to 13 cell layers of new tissue but relatively slight growth of the uterine epithelium.

Summary. This preliminary study, utilizing recently developed fluorimetric technics

³ Cohen, H., and Bates, R. W., *J. Clin. Endocrinology*, 1947, 7, 701.

⁴ Finkelstein, M., Hestrin, S., and Koch, W., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 64.

⁵ Bates, R. W., and Cohen, H., *Fed. Proc.*, 1947, 6, 236.

⁶ Jailer, J. W., *Endocrinology*, 1947, 41, 198.

together with standard biological procedures, indicates that the eggs of the American lobster at the time of attachment to the caudal appendage contain estrogenic activity estimated at a minimum of 100 international units per

100 g of eggs. That a substantially increased yield may be affected by further refinements in extraction methods is now being investigated.

16661

Microdetermination of Steroid Estrogens in Urine by Fluorometry.*

M. FINKELSTEIN. (Introduced by Bernhard Zondek.)

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We have previously described¹ a fluorometric method for determination of estradiol, estrone and estriol in pure solution, which was much more sensitive and accurate than the colorimetric methods used in determining these substances. The fluorometric method was only slightly less sensitive for estradiol and estrone than the biological tests, while estriol could be determined at levels too low for biological assay. In all cases the accuracy of the fluorometric method exceeded that of the biological method and the range of error was found to be $\pm 3\%$.

The specificity of the new reaction was high, only very few substances interfering with the determination of the estrogens; and since none of these were phenols they could easily be separated from the estrogens and therefore represented no problem in the assay.

The present paper deals with adaptation of the fluorometric method to determination of estradiol, estrone and estriol in urine.

Experimental. (With the assistance of Mrs. B. Wolman.) The following apparatus and reagents were employed in the tests:

1. Coleman Electronic Photofluorometer, Model 12A, with filters B₂, B₁ (optical), PC₂ and PC₁.

2. All-glass distillation apparatus.

* We are deeply indebted to Dr. B. Y. Brent for supplying us with estradiol and estrone, and to Dr. Oliver Kamm for estriol.

¹ Finkelstein, M., Hestrin, S., and Koch, W., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 64.

3. Test tubes with ground glass stoppers (Pyrex, 15 ccm).

4. Reagents.

- a. Ether, peroxide, free, redistilled.
- b. Sodium bicarbonate, anhydrous, pro anal.
- c. Sodium carbonate, anhydrous, pro anal.
- d. Hydrochloric acid, concentrated, pro anal.
- e. Sulfuric acid, pro anal., diluted with distilled water 4:5.
- f. Sodium hydroxide, pro anal.
- g. Ethanol, redistilled.
- h. Benzene, triophene free.
- i. Phosphoric acid, purest, s.g. 1.75.
- j. Sodium chloride, pure.
- k. Crystalline estrogens.[†]

A. *Determining the standard estrogen curves.* The Coleman fluorometer provided with the B₂ primary filter and the PC₂ secondary filter was calibrated with 7 cc of a sodium fluoresceinate solution (0.05 γ / cc), so that the value 80 was recovered on the galvanometer scale.

Crystalline estradiol, estrone and estriol dissolved in pure ethanol 96% were used in preparing the standard curves. Substantially smaller amounts of estrogen were used in determining the standard curve than in our previous study,¹ as the response in the Coleman fluorometer was much higher than

[†] We are deeply indebted to Dr. B. Y. Brent for supplying us with estradiol and estrone, and to Dr. Oliver Kamm for estriol.

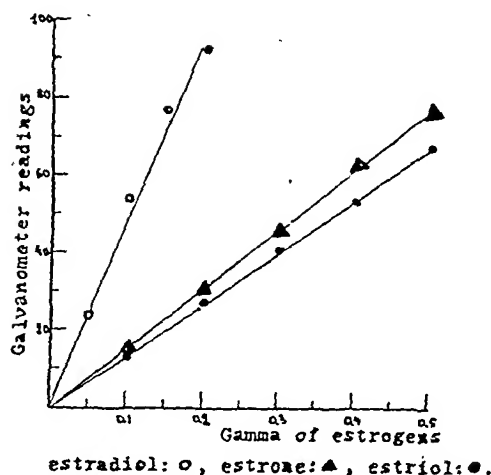


FIG. 1.
The standard curve of estrogens.

that obtained in the Lumetron fluorometer previously employed. The curves of estradiol, estrone and estriol were linear throughout the scale and the limits were 0.05 γ to 0.20 γ for estradiol and 0.1 γ to 0.6 γ for estrone and estriol.

Our procedure in preparing the standard curves was essentially similar to that in our earlier paper, except that 7 cc rather than 2.5 cc of phosphoric acid was used in developing and reading the fluorescence. The standard curves are presented in Fig. 1.

B. Fractionation of urine in the estradiol-estrone and estriol fraction. We generally followed the method of Bachmann & Pettit² in fractionation though some of the steps in the original method were modified and simplified. Only 10 cc of urine were required in our method, compared with 100 cc in the original method. The recovery percentages, however, fell within the same limits as those described by Bachmann & Pettit.² The procedure was as follows:

10 cc of fresh urine were rendered acid with 0.7 cc concentrated HCl (pro anal.) and hydrolyzed under reflux for 1 hour in an all-glass apparatus. The urine was then cooled under running tap water, 5.6 g of sodium chloride was added to it, and it was diluted with 10 cc of distilled water. The

solution was extracted 5 times with 20 cc of benzene in a separatory funnel. The pooled benzene extracts (100 cc) were washed in the funnel with 3 cc of sodium bicarbonate 9%, following which the benzene was concentrated to 30 cc. The benzene extract was then shaken in a separatory funnel once with 30 cc and twice with 15 cc of sodium carbonate 9%, and once with 7 cc of water; and the aqueous washings and the sodium carbonate extracts were pooled. Following this procedure all the estradiol and estrone remained in the benzene fraction, while the estriol was almost quantitatively removed in the sodium carbonate fraction. From this point on the two fractions were treated separately.

The estriol fraction. The pooled carbonate extract was rendered acid (less than pH 6) with concentrated HCl and then 3 times extracted with 35 cc of ether. The pooled ether extracts were washed twice in a clean separatory funnel with 10 cc of sodium bicarbonate 9% and twice with 10 cc of water. The ether was then evaporated and the residue was taken up in a measured volume of ethanol, between 10 and 20 cc, and aliquots of this solution were used for fluorometry. In cases when the ether residue was colored, the following additional purification was employed. The residue was taken up in 0.5 cc of ethanol and the solution was diluted with 50 cc of benzene, washed with 2 cc of sodium bicarbonate 9%, and extracted 3 times with half its volume of 0.01 N NaOH and once with 10 cc of water. The pooled extracts were made acid with concentrated HCl and extracted 3 times with half their volume of ether. The ether was evaporated to dryness and the residue was taken up in a measured volume of ethanol, and aliquots of this solution were used for fluorometry.

The estradiol-estrone fraction. The concentrated benzene extract was washed once with a quarter of its volume of dilute sulfuric acid and twice with 15 cc of water. It was then extracted 4 times with an equal volume of N NaOH. The pooled alkaline extracts were acidified with concentrated HCl (pH less than 5) and were then extracted 3 times with 50 cc of ether. The combined

² Bachmann, G., and Pettit, D. S., *J. Biol. Chem.*, 1941, 138, 689.

TABLE I.
Recovery of Estradiol, Estrone or Estriol from 10 cc Urine Samples.

Estrogen added	γ of estrogen added	γ of estrogen observed after subtraction of the blank value	% of recovery
Estradiol	1	0.6	60
"	10	8	80
"	100	84	84
Estrone	50	37	74
"	50	35	70
"	100	80	80
Estriol	100	56	56
"	100	42	42
"	50	28	56
"	50	30.5	61
"	25	12.5	50
"	25	17	68

extracts were concentrated to about 50 cc, washed once with 10 cc of dilute sulfuric acid, twice with 20 cc of sodium carbonate 9% and twice with 20 cc of water. The ether extract was evaporated, the residue was taken up in a measured volume of ethanol, and aliquots of this solution were used for fluorometry.

C. *The fluorometric measurement.* Aliquots in Pyrex test tubes with ground glass stoppers were evaporated to dryness in an electric oven at about 120°C with the stoppers removed. After cooling, 7 cc of phosphoric acid were added to each, the tubes were stoppered and heated in a boiling water bath in the dark for half an hour. When the tubes were cooled, the fluorescence was measured in the Coleman fluorometer, using filters B₂ + PC₂. For blank readings the measurement was repeated using filter B₁ (optical) + PC₂, and this value was subtracted from the first readings.

Results. In the recovery experiments the tested estrogen was added to 10 cc of hydrolyzed urine. A blank experiment for this sample was run at the same time, and the value thus obtained was subtracted from the experimental reading. Table I summarizes the results of the recovery experiments.

As may be seen from the table, the best estrogen recoveries (above 80%) were obtained with estradiol. As little as 1 γ of estradiol added to 10 cc of urine could be demonstrated with 60% recovery. The recoveries of

estrone were in the range of 70%, and those of estriol were lower, between 50 and 60%. Since an inhibitory effect was observed when concentrated aliquots of the estriol fraction were used, we recommend the use of diluted samples of this estrogen for fluorometric assay.

Discussion. The present study describes an accurate and time-saving method for the determination of urinary estrogens, which is also specific and is not influenced by substances accidentally occurring in the urine. Its particular advantage is its sensitivity, which permits the use of a urine sample as small as 10 cc of normal urine and even less of pregnancy urine. The fluorometric readings are not complicated by interfering substances in the urine, as are the colorimetric readings according to Kober.³ Thus the estrogen concentration may be calculated in a single reading. Recently, Jailer⁴ adapted our fluorometric method to the analysis of estrogens in urine. In a preliminary study he reported the recovery of estrone and estradiol, using diluted sulfuric acid instead of phosphoric acid. This modification, however, proved to be of less value than the original method, since estriol was not detectable by this method, and since his recoveries of estradiol and estrone were in the range of 50%. Furthermore, some of the sterolic substances which did not fluoresce when phos-

³ Kober, S., *Biochem. J.*, 1938, **32**, 357.

⁴ Jailer, W., *Endocrinology*, 1947, **40**, 198.

phoric acid was used yielded fluorescent products with sulfuric acid.

A detailed study of metabolism of estrogens in normal, pregnant and pathological conditions will be published elsewhere.

Summary. 1. A quantitative method for the determination of estradiol, estrone and estriol in urine, based on the fluorescence of these substances when treated with phosphoric acid, is described. 2. The method is more accurate, sensitive and specific than other known

biological or colorimetric tests. 3. The recoveries of estradiol were above 80%; of estrone between 70 and 80%; and of estriol between 50 and 60%. The mean experimental error was $\pm 10\%$. 4. The described method permits the determination of estriol in urine in quantities not hitherto detectable.

The author wishes to express his indebtedness to Prof. Bernhard Zondek for his kind interest and support of this investigation.

16662

Isotopic Studies of Fixation by Rhizobia in Presence of Hemoprotein.*

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Claims of fixation by free-living species of *Rhizobium* in the presence of plant extracts, growth factors and other organic supplements frequently have been made but not verified.¹ The most recent, that hemoprotein from the root nodule can induce fixation *in vitro*, was suggested by the observation that the pigment occurs in the red state during active fixation but disappears from ineffective or old nodules. Although attempts to substantiate the positive experiments failed,^{2,3} such negative findings are not necessarily critical. Fixation by pure cultures might be stopped by the accumulation of an intermediate, and pure cultures, long cultivated on combined nitrogen in the laboratory, might lose their ability to assimilate molecular nitrogen. These objections can be overcome by testing for fixation with the stable isotope, N^{15} , and by using species of

Rhizobium taken directly from the nodules. Whereas the conventional Kjeldahl method requires fixation of 1.0 mg or more for positive claims, the isotopic method readily detects fixation of as little as 0.01 mg and practically eliminates sampling errors.

Materials and Methods. By "pigment" is meant an aqueous extract from nodules actively fixing N_2 and red with hemoprotein. This extract, after filtration through cheesecloth and centrifugation in a Beams centrifuge, is sterilized by passage under pressure through a Seitz filter. The preparations are usually gummy and difficult to filter. In initial experiments with such filtrates fixation was obtained. In agreement with our earlier results² the increases in nitrogen content were too small to have been detected by the Kjeldahl method, but were definite with the isotopic technic. Microscopic and cultural tests revealed a large number of organisms, primarily the root nodule bacteria. Inability to secure sterile filtrates did not arise from faulty technic or poor filters since no difficulty was experienced with laboratory cultures of the rhizobia. These observations suggest a sub-microscopic filterable form in the nodule (Almon and Baldwin).⁴ We believe, however, it is more likely that the effect

* Supported in part by grants from the Rockefeller Foundation and from the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation.

1 Wilson, P. W., *The Biochemistry of Symbiotic Nitrogen Fixation*. University of Wisconsin Press, 1940.

2 Niss, H. F., and Wilson, P. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 233.

3 Virtanen, A. I., Jorma, J., Linkola, H., and Linnasalmi, A., *Acta Chem. Scand.*, 1947, **1**, 90.

TABLE I.
Summary of Isotopic Nitrogen Fixation Experiments.

Exp.	Source of pigment	Cultures	Atom % excess N ¹⁵		
			Initial gas	Inoculated	Uninoculated
1	Sterile Soybean, cowpea	534	8.85	.010	— .001
2	Cowpea	317, 618	10.20	.011	
		<i>Azotobacter</i>		3.5	
		<i>Clostridium</i> *		.36	
		<i>Radiobacter</i>		.009	
		Mixture†		.90	
3	Soybean	317 and			
		<i>Radiobacter</i>	10.80	.010	
4	"	317, 610, 534		— .016	
		and <i>Radiobacter</i>	10.60	— .016	
5	"	<i>Clostridium</i> *	8.73	.013	
				.011	
6	Nonsterile Soybean	534	7.88	.045	.062
				.151	.053
7	"	317, 534	2.84	.040	.048
				— .009	
				— .001	
				— .020	
8	Cowpea	317	8.15	— .001	— .001
				— .002	— .004
9	"	610	9.40	.012	.014
10	Soybean		10.06		.076
					.076

Increase of 0.050 atom % excess necessary for significant gain.

* Isolated from a filtered but nonsterile nodular extract.

† Mixture of all cultures used in experiment.

of the gum on the physical properties of the filter determines whether a given organism is retained since suitable dilution of the gummy preparation resulted in a sterile filtrate.

This curious filtering property of the root nodule bacteria was unexpected but useful as it provided a convenient method for securing a culture of the organism directly from the nodules for test of its ability to fix nitrogen. The uncertainty of whether other contaminating forms likewise accompanied these "filterable" rhizobia necessitated suitable bacteriological observations for control of the population in such nonsterile cultures. Microscopic stains were made at every stage of the preparation of the pigment to note the decrease in numbers of organisms, and cultural tests were made on the contents of the isotopic flasks at the end of the experiment. *Azotobacter* was tested for by inoculation of Burk's nitrogen-free medium, *Clostridium*, by inoculation of

Winogradsky's nitrogen-free medium and incubating in an atmosphere of molecular nitrogen; and *Rhizobium* by plating on yeast water-mineral salts—sucrose agar. All media were incubated for at least two weeks before discarding; if any growth occurred, a microscopic examination was made; if fixation, the culture was further identified.

The species of *Rhizobium* used were: pea culture 317, soybean culture 534, cowpea cultures 610 and 618. Both positive and negative controls were obtained by including *Azotobacter vinelandii*, a species of *Clostridium* isolated from a soybean nodule, and *Alcaligenes radiobacter*. Details of the specific isotopic technics are given by Burris, Eppling, Wahlin, and Wilson.⁵ Incubation in the presence of the N¹⁵ was for 48 hours at 24-29°C.

Results and Discussion. Results from typical experiments are summarized in Table I. The outstanding conclusion is that none

⁴ Almon, L., and Baldwin, I. L., *J. Bact.*, 1933, 20, 229.

⁵ Burris, R. H., Eppling, F. J., Wahlin, H. B., and Wilson, P. W., *J. Biol. Chem.*, 1943, 148, 349.

of the sterile preparations brought about fixation by the rhizobia or by mixtures of rhizobia and radiobacter, a frequent contaminant of nodules. The positive results with *Azotobacter* and *Clostridium* demonstrate that fixation could occur under the experimental conditions. As shown in *Experiment 5* fixation is not always obtained with *Clostridium* probably because of the presence of free oxygen and soluble organic nitrogen. Neither of these would prevent fixation by *Azotobacter*. To reduce the level of soluble available nitrogen, one experiment was made using a purified hemoprotein solution and also hemoglobin from blood. Neither induced fixation by the rhizobia, nor did the addition of oxalacetate alter this result.

Nonsterile preparations did not consistently induce fixation, the positive results being concentrated in two experiments. The results with the nonsterile filtrates are significant for the argument that lack of fixation by pure cultures of the rhizobia arises through loss of this physiological function after laboratory cultivation. In these experiments preparations were obtained that, although filtered, contained numerous rhizobia. These were

identified by microscopic examination, cultural characteristics, physiological tests in litmus milk and veal infusion broth, and by inoculation of the host plant. Such organisms, tested for fixation immediately after recovery from the nodule and prior to any transfer on artificial medium, were unable to fix even the minute quantity of nitrogen that can be detected by the isotopic method (*Experiment 7, 8, and 9*). In the positive trials with nonsterile filtrates (*Experiment 6 and 10*) species of *Clostridium* demonstrated to be capable of fixing nitrogen were isolated.

Summary. When tested by the sensitive isotopic method, neither an aqueous extract of nodules nor a purified preparation of hemoprotein could induce fixation of atmospheric nitrogen by free-living *Rhizobium*.

Cultures of the root nodule bacteria taken directly from the nodules and tested prior to cultivation on laboratory media did not fix nitrogen even though hemoprotein was present.

In a few experiments, fixation of molecular nitrogen was obtained, but the nodular preparations were shown to contain nitrogen-fixing clostridia.

16663

Effect *in vitro* of Specific Lipid Fractions of Animal Sera on Psittacosis Virus.

JOHN P. UTZ. (Introduced by Charles Armstrong.)

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Casals and Olitsky¹ have reported that a number of neurotropic viruses were inactivated when mixed with various animal sera and incubated at 37°C. Furthermore when these sera were successively extracted with acetone, ether, and hot alcohol-ether, each extract was effective in inactivating virus. This report attempts to confirm these results—using psittacosis virus—and to isolate that lipid fraction having the virus-inactivating property.

Method and materials. Virus: Psittacosis virus originally isolated by Dr. Dorland Davis from a fatal disease in a parrot ("4" strain) was propagated in the allantoic sac of 8-day-old chick embryos. Allantoic fluid was

TABLE I.
Fifty Percent End-point of Psittacosis Virus Incubated at 37° in Presence of Serum, Saline and Sterile Water.

	1 hr	2 hr	4 hr	24 hr
Serum	10-6.0	10-6.0	10-5.8	10-3.6
Water	10-6.0	10-6.0	10-4.2	10-1.1
Saline	10-6.1	10-5.8	10-2.4	10-0

¹ Casals, J., and Olitsky, P. K., *Science*, 1947, 106, 267.

TABLE II.

Average of 50% End-points a Single Pool of the "4" Strain of Psittacosis Virus Incubated for 1 Hour at 37°C in Presence of Serum and Egg Yolk Extracts and Lecithin.

Flow sheet	Avg of LD ₅₀ of psittacosis in serum extr.	Avg of LD ₅₀ of psittacosis in egg yolk extr.	Avg of LD ₅₀ of egg yolk lecithin*	Avg of LD ₅₀ of vegetable lecithin†
Serum or egg yolk -----	10 ^{-6.1}	10 ^{-6.0}		
Bloor's reagent				
filtrate	10 ^{-6.2}	10 ^{-6.1}		
petrol ether				
ppte				
filtrate ----- total lipid -----	10 ^{-4.0}	10 ^{-5.8}		
acetone				
filtrate				
MgCl ₂				
ppte	10 ^{-4.3}	10 ^{-6.1}		
filtrate-----phospholipid free-----				
ether				
ppte-----sphingomyelin-cerebroside -----	10 ^{-4.6}			
filtrate ----- lecithin cephalin -----	10 ^{-1.3}	10 ^{-5.8}		
CdCl ₂				
ppte				
filtrate ----- cephalin-like -----	10 ^{-1.8}			
NH ₃				
filtrate-----lecithin-like -----	10 ^{-0.8}			
Control of egg and vegetable lecithin -----			10 ^{-5.9}	10 ^{-6.0}

* Mr. Theriault of National Institute of Health.

† Pfanstiehl vegetable lecithin.

pooled, cultured for contaminants, titrated in mice and quick frozen in sterile ampules in 2 cc lots. The 6BC and Gleason strains of psittacosis virus obtained from Dr. Orville Golub of Camp Detrick were also used.

Serum extracts. Total lipid extracts by Man's method² were made of sera from normal chicken, rabbit, horse, sheep and human bloods. The petroleum ether solution of the serum lipids was then concentrated under negative pressure of 28 inches of mercury at a temperature of 35-40°C in a constant small stream of dry nitrogen. Seven volumes of acetone were then added to this concentrated extract and allowed to stand overnight at 4°C. The precipitate was centrifuged at 1500 r.p.m. in a standard centrifuge for 5 minutes, the supernatant poured off and to it was added 1 drop of a saturated solution of magnesium chloride.³ This was

again centrifuged and the supernatant was saved as the phospholipid-free fraction of the total lipid constituents of serum. The precipitate in acetone was then divided into the ether-soluble and ether-insoluble fractions, the latter being saved as the sphingomyelin-cerebroside fractions of serum. The lecithin-like fraction was isolated from the ether-soluble fraction using Maltaner's⁴ modification of the technic of Levene and Rolf.⁵ The cephalin-like fraction was recovered in the ether washing of the cadmium lecithin. Each of these 4 fractions—phospholipid-free, sphingomyelin-cerebroside, lecithin and cephalin—was concentrated and to each was added an amount of sterile distilled water

³ Sinclair, R. G., Dolan, M., *J. Biol. Chem.*, 1942, 142, 659.

⁴ Maltaner, F., *JACS*, 1930, 52, 1718.

⁵ Levene, P. A., and Rolf, I., *J. Biol. Chem.*, 1927, 72, 587.

² Man, E. B., *J. Biol. Chem.*, 1937, 117, 183.

equal to the amount of original serum. The organic solvent was then bubbled off with nitrogen gas. The extracts were in some cases dried completely with nitrogen gas and then taken up with water with no difference in the final titer. No further steps were taken to identify the fractions.⁶ Except for the manifestly-necessary last step, great care was taken to avoid exposure to air, temperatures over 40°C, and water.

Titration. The infected allantoic fluids were made up in 10-fold dilutions in each of the above extracts as well as in controls of distilled water, saline, non-infected allantoic fluids and egg yolk extracts. After incubation at 37°C for times varying from 1-24 hours, 0.03 cc were injected intracranially into 6-week-old mice of the NIH stock strain (10-20 mice for each dilution), and the LD₅₀ computed by the method of Reed and Muench.

Results. Since the virus was gradually inactivated in any diluent when incubated at 37°C for longer than 4 hours (Table I), the various fractions were compared on the basis of one-hour results. Under these conditions no virus inactivation could be demonstrated by normal sera. Virus-inactivating properties could be demonstrated, however, in lipid extracts of the same sera. Table II, presenting the averages of 15 extractions, demonstrates that the phospholipid-free and sphingomyelin-cerebroside fractions have a 10-100 fold inactivating effect, that the lecithin-like and cephalin-like fractions decreased the titer 10,000-100,000 fold, and that the lecithin-like fraction was about 10 times more effective than the cephalin-like fraction. Comparable results were obtained with the 6BC and Gleason strains. Controls of water; saline; egg yolk extracts; commercial lecithin, prepared in concentration equal to that expected in serum extracts by bubbling off the organic solvent in the presence of water; all failed to exert any such inactivating effect.

Discussion. This work fails to demonstrate that animal sera can inactivate psittacosis virus, but tends to confirm findings of others¹ that virus inactivation can occur in isolated lipid fractions peculiar to serum. It should be pointed out that different yields with different titres were frequently obtained from

TABLE III.
Average of 50% End-points of a Single Pool of the "4" Strain of Psittacosis Virus Incubated for 1 Hour at 37° in the Presence of Serum and Egg Yolk Extracts and Lecithin.

	Chicken	Sheep	Sheep	Human	Chicken	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Horse	Pooled	Pooled	Avg	Egg yolk	Egg yolk
	1	2	1	1	2	3	4	4	5	5	6					1	2
Serum	10-6.0	10-5.8	10-6.1	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	10-6.1	10-6.2	10-5.8
Denatured																	
Protein	10-6.1	10-6.2	10-5.9												10-6.2	10-6.3	10-5.8
Total lipid	10-1.0	10-4.0	10-4.1		(2)										10-4.0	10-5.0	10-5.7
Phospholipid free	10-4.0	10-3.0	10-4.3	10-3.5	10-4.5	10-4.1	10-1.5	10-5.0	10-4.6	10-5.2	10-5.9	10-4.8	10-4.6	10-4.3	10-4.3	10-6.2	10-6.0
Sphingomyelin																	
cerebroside	10-1.6	10-5.1	10-3.8												10-4.6		
Cephalin																	
lecithin	10-1.5	10-3.0	10-4.8	10-2.9	10-4.0	10-1.5	10-0	10-0	10-0	10-0	10-0	10-3.0	10-1.8	10-1.5	10-1.3	10-6.0	10-5.6
Cephalin																	
lecithin																	
Egg yolk																	
lecithin																	
Vegetable																	
lecithin																	

(1) Greater than 10-4; 100% mortality at 10-4.
(2) Greater than 10-6; 80% mortality at 10-6.
(3) Greater than 10-5; 90% mortality at 10-5.

equal aliquots of the same sera. This may be due to the difficulties in extracting a pure substance that is not contaminated or decomposed.⁶

Summary. Specific lipid fractions of cer-

tain animal sera appear capable of inactivating psittacosis virus *in vitro* though the original sera exerts no such effect. The virus inactivating property appears to be in the lecithin-like and cephalin-like fractions, and peculiar to serum lipids.

⁶Bull, H. B., *Biochemistry of the Lipids*, 1937.

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16664 P

Growth of Hypophysectomized Rats Injected with Growth Hormone and Fed on Different Proteins.

BACON F. CHOW AND ROY O. GREEP.

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A few studies¹⁻⁵ dealing with the relationship between the hypophysis and nutrition have been reported, but no systematic investigation on the nutritional requirements of hypophysectomized rats has yet been made. In this communication are recorded our preliminary results of the growth studies of hypophysectomized rats fed with proteins of different nutritive values.

Method. The procedure for the growth study is essentially that of Osborne and Mendel.⁶ Five groups (10 animals each) of hypophysectomized rats were fed isocaloric diets containing 30% test proteins, 32% Amidex,* 24% Crisco, 2% Cod Liver Oil, 8% yeast, and 4% of a salt mixture. The vitamin supplements for each one hundred grams of the diet are:

¹ Lee, M. O., and Ayres, G. B., *Endocrinology*, 1936, 20, 489.

² Samuels, L. T., Reinecke, R. M., and Bauman, K. L., *Endocrinology*, 1943, 33, 87.

³ Samuels, L. T., *Recent Progress in Hormone Research*, 1947, 1, 147.

⁴ Gordan, G. S., Bennett, L. L., Li, C. H., and Evans, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1947, 65, 317.

⁵ Gordan, G. S., Bennett, L. L., Li, C. H., and Evans, H. M., *Endocrinology*, 1948, 42, 153.

	mg
Thiamine HCl	0.3
Riboflavin	0.3
Niacin	2.0
Pyridoxine HCl	0.2
Calcium pantothenate	2.0
Choline chloride	100.0

⁶ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1926, 69, 661.

* Amidex is dextrine obtained from Corn Products Refining Co. of N.Y.

Biotin	0.01
Folic acid	0.025
Inositol	10.0
p-Aminobenzoic acid	25.0

The food consumption and the weight of the test animals were recorded twice weekly. During the entire test period of three weeks, the animals received subcutaneously 0.1 mg of a purified growth hormone preparation, isolated from beef pituitary glands; every morning.

Results. The data (see the accompanying table) demonstrate that animals on a protein-free diet failed to survive for even a week in spite of their average consumption of approximately 5 g per rat per day. In contrast with this the normal, unoperated rats of the same age survived for more than 2 weeks on the same protein-free diet in spite of their gradual loss of weight. The weights of animals on the test protein diets increased significantly. The increase was the smallest for soya protein diet and the greatest for the casein diet, even though the animals on soya protein consumed approximately the same amount of food as the animals on either the vitamin-free casein or lactalbumin diets. After the second and third weeks of feeding, the food consumption by rats of all groups dropped markedly. The increase in body weights was likewise less pronounced than that of the first week and could not be accentuated even when another 0.1 mg of

growth hormone was injected late in the afternoon. This seems to indicate that the submaximal growth is not due to the insufficiency of growth hormone.

Our preliminary data therefore leads us to believe that soya protein, which has been demonstrated to be inferior for normal rats, did not support the growth of hypophysectomized rats as well as the proteins of higher quality, such as casein and lactalbumin. However, in this experiment the superiority of lactalbumin over casein, so often demonstrated in the maintenance of or in the promotion of the growth of normal rats, failed to reveal itself in the growth of the hypophysectomized rats.

It has been demonstrated that hyperthyroidism⁷⁻⁹ in rats may increase the requirements of some of the known vitamins and perhaps some unknown nutrient factors present in liver or yeast.

Since our growth hormone preparation was most likely contaminated with thyrotropic hormone, it is possible that our observed difference in the growth promoting properties of the samples of proteins may be explained not on the basis of the amino acid content but on the contaminating nutritional factors in the test proteins. In any case, our data demonstrate that the quality of proteins in the diet can affect the growth of hypophysectomized rats, and this factor must now be emphasized in the assay of growth hormone preparations.

TABLE I.
Food Consumption and Body Weights of Hypophysectomized Rats Injected with Growth Hormone and Fed Different Proteins.

Protein fed	Weeks of feeding							
	0		1		2		3	
	F.C.*	Avg wt in g	F.C.*	Avg wt in g	F.C.*	Avg wt in g	F.C.*	Avg wt in g
Protein-free	0	43.7	35	40.0	—	—	—	—
Soya protein	0	45.9	44	55.9	26	57.7	7.6	59.4
Crude casein	0	47.1	56	63.2	30	68.5	34	71.9
SMA vit.-free casein	0	45.5	47	61.3	24	66.3	25	67.5
Lactalbumin	0	44.8	40	58.9	22	64.0	20	68.8

* F.C. = average weight in grams of food consumed per rat per week.

⁷ Bethell, J. J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutrition*, 1947, **34**, 431.

⁸ Ershoff, B. H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 500.

⁹ Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 400.

Influence of Age and Mammary Development upon Mammary Carcinogenesis Following Injection of "Milk Agent."*

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It has been observed that when mice of a genetically high tumor strain but lacking the milk agent are infected with this virus after they are a few months of age they develop a much lower incidence of mammary carcinoma than if infection occurs when they are under a month of age.^{1,2} Because of this observation and that of Van Gulik and Korteweg³ that the degree of lateral bud formation in the mammary gland is determined by the presence or absence of the milk agent, it was suggested⁴ that in order to be effective in the genesis of mammary cancer the milk agent virus has to be present at the time the mammary gland develops. More recently Dmochowski⁵ has been able to overcome this relative "non-susceptibility" of older mice by increasing the amount of virus injected and by injecting it weekly over a 3 month period. This, together with our inability to confirm the above mentioned observations of Van Gulik and Korteweg,⁶ prompted the present investigation. Since the mammae of male mice remain essentially rudimentary throughout life, the demonstration that castrate male

mice bearing ovarian grafts may develop a high incidence of mammary cancer⁷ suggested a suitable method for testing the relationship of the state of mammary gland development at the time of the introduction of the milk agent to mammary carcinogenesis.

Materials and methods. Male mice of the Zb stock (fostered C₃H), being genetically susceptible to the development of breast cancer but lacking the milk agent, were used for this experiment since their mammae normally show almost no development throughout life (less than those of A x Z hybrid males). Three groups of mice were employed. In the first, castration and ovarian transplantation were performed when the mice were approximately one month of age, and on the following day they were injected intraperitoneally with an extract of a spontaneous mammary tumor containing the milk agent. In the second, castration and ovarian transplantation were carried out when the animals were one month of age, but the injection of milk agent virus was postponed until the animals were 4 months of age in order to allow mammary gland development prior to the introduction of the virus. In the third group, castration and ovarian transplantation were performed when the mice were 4 months of age, and on the following day the animals were injected with a tumor extract. In the second and third groups, the animals received the milk agent when approximately the same age, but in Group 2 the mammae had developed prior to the introduction of the virus while in Group 3 mammary gland development occurred in the presence of the milk agent.

The methods of castration and ovarian transplantation were the same as described in

* Assisted by grants from the Minnesota Division of the American Cancer Society, the Citizens Aid Society of Minneapolis, the Graduate School Cancer Research Fund of the University of Minnesota, and the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

¹ Duran-Reynals, F., and Bittner, J. J., quoted by Bittner, J. J., *Cancer Research*, 1942, **2**, 710.

² Andervont, H. B., Shimkin, M. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1942, **3**, 309.

³ van Gulik, P. J., and Korteweg, R., *Proc. Nederl. Akad. v. Wetenschappen*, 1940, **43**, 891.

⁴ Bittner, J. J., *Cancer Res.*, 1942, **2**, 710.

⁵ Dmochowski, L., *Brit. J. Exp. Path.*, 1945, **26**, 192.

⁶ Huseby, R. A., and Bittner, J. J., *Cancer Res.*, 1946, **6**, 240.

⁷ Huseby, R. A., and Bittner, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1948, **60**, 321.

the previous paper, and ovaries of one month old Zb females were used in all instances. To obtain active virus preparations, fresh mammary tumors arising spontaneously in Z strain females were employed. The tumors were ground in a mortar with sand, extracted with distilled water, and centrifuged at relatively low speeds to remove viable cells, as well as larger cellular and connective tissue fragments. Each animal received 1 cc of a 10% extract intraperitoneally. The animals were maintained on Purina Fox Chow, and inspected weekly for the development of subcutaneous tumors. All animals developing such tumors were autopsied.

Results. As a preliminary study to determine the extent of glandular development at the time of the introduction of the milk agent in the second group of experimental animals, glands of Zb mice castrated and transplanted with ovaries at a month of age were studied histologically when the mice were 4 months of age. From the study of whole mount preparations it was evident that by this time several of the glands of each animal had developed extensively (all 10 glands seldom develop in male mice in response to estrogenic stimulation). The ducts of the developed glands had extended throughout the fat pads to a degree similar to that seen in virgin female mice of the same age. In addition to ductular and lateral bud development, however, there were many areas of alveolar development, a situation not encountered in virgin female mice lacking the milk agent. These areas occurred mainly near the periphery of the glands and resembled normal alveolar lobules as seen in pregnant mice approaching parturition. Morphologically they differed from the typical precancerous lesions seen in glands of high tumor strains of mice in that the epithelium showed considerable secretory activity, and there was not the slight increase of connective tissue usually seen in precancerous hyperplastic nodules. Because of the presence of these areas of alveolar development, however, a fourth group of Zb males were castrated and transplanted with ovaries when one month of age, but they were not injected with the milk agent

Summary of Tumor Data Obtained in the Various Groups of Male Mice That Were Castrated, Transplanted with Ovaries, and Injected with Milk Agent Virus at Different Ages.

Group	Age when transplanted with ovaries, mo.	Age when injected with milk agent, mo.	No. of animals/ No. tumorous	% tumorous		Avg age of tumor development		Avg age of non-tumorous death, mo.
				uncorr.	corr.	from birth, mo.	from inject. of milk agent, mo.	
I	1							
II	1	1	34/25	73.5	78.1	11.5	10.5	14.8
III	4	4	30/8	26.7	32.2	14.0	10.7	17.1
IV	1	None injected	34/8	23.5	29.1	15.1	10.7	16.8
			16/0	0	0	—	—	20.1

virus in order to make certain that mammary tumors would not develop in such lobules in the absence of the virus.

The mammae of male mice castrated and transplanted with ovaries when four months of age were also studied three months after transplantation to determine if their glands had developed to the same extent as those of the younger mice. As far as could be ascertained from the inspection of whole mount preparations, this was the case.

The tumor data are summarized in Table I. In the group receiving both ovaries and milk agent at one month of age, mammary tumors developed at an earlier average age than in virgin females of the Z strain maintained in this laboratory⁸ confirming the observations previously reported employing hybrid males.⁷ In the 2 groups that received the milk agent at 4 months of age the incidence of mammary tumors was only about one third that seen in the group that received virus when one month of age, but there would appear to be no difference in the age at which tumors developed or in the frequency of tumor development in these latter 2 groups of animals. It is interesting that the average latent period of tumor development after the injection of the milk agent was the same in all 3 groups of animals.

Since the age distribution of tumor development after the injection of milk agent was the same for all 3 groups of mice, it would appear that the correction factor developed by Bryan and Shimkin⁹ would be applicable to these data and would give some indication whether or not the lower incidence of tumors observed in the 2 groups of mice injected at 4 months of age was entirely due to the fact that these animals had a somewhat shorter time in which to develop cancer. The per cent of tumors developing during each 30 day period following the injection of the milk agent was calculated, and the mice dying non-tumorous weighted accordingly. As can be seen from the "corrected" data given in

the sixth column of Table I, only a small portion of these lower incidences of tumors can be attributed to differences in the life span following the injection of the virus.

Discussion. The results of this experiment emphasize again that as they grow older genetically susceptible mice that lack the milk agent become less "susceptible" to the milk agent virus, at least as far as the subsequent development of mammary tumors is concerned. These data would also seem to indicate that this relative "non-susceptibility" is not due to the greater mammary gland development in female mice after one month of age since glands of 4 month old male mice that developed after the injection of milk agent had no more tumors than did glands that developed prior to the introduction of the virus.

It has been shown that young mice are more susceptible to chemical and physical carcinogens than are older mice.¹⁰⁻¹³ As yet we have been unable to demonstrate in older mice lacking the milk agent a humoral agent capable of neutralizing active milk agent virus either *in vitro* or *in vivo*. It has been observed, furthermore, that although mice which obtain the milk agent late in life may die non-cancerous, such mice may pass the agent to their offspring which then may show a high incidence of tumors. This relative "non-susceptibility" to the development of mammary tumors following the late introduction of the milk agent may not, then, be a specific resistance to the virus but may reflect a more general increased resistance of older mice to the influence of "carcinogens." Studies are now under way in an attempt to shed more light on this possibility.

Summary. Employing male mice, transplanting ovaries and injecting active milk agent virus at different ages, an experiment

¹⁰ Mider, G. B., and Morton, J. J., *Am. J. Cancer*, 1939, **37**, 355.

¹¹ Cowdry, E. V., and Suntzeff, V., *Yale J. Biol. and Med.*, 1944, **17**, 47.

¹² Kaplan, H. S., *Cancer Res.*, 1947, **7**, 141.

¹³ Smith, W. E., and Rous, P., paper presented at the 39th Annual Meeting of the Amer. Assn. for Cancer Res., 1948.

⁸ Bittner, J. J., and Huseby, R. A., *Cancer Res.*, 1946, **6**, 235.

⁹ Bryan, W. R., and Shimkin, M. B., *J. Nat. Cancer Inst.*, 1941, **1**, 807.

was carried out to specifically test the relationship of the state of mammary gland development at the time of the introduction of the milk agent to mammary carcinogenesis.

The results would indicate that the relative non-susceptibility of older mice is not due to greater mammary gland development at the time of virus infection.

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Effects of Excess Dietary *dl*-Methionine and/or *l*-Arginine on Rats.*

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The addition of low concentrations of *dl*-methionine to a casein diet increases the nitrogen balance index in the rat while the addition of higher concentrations of the amino acid decreases the index and causes a marked loss in body weight.^{1,2} Loss in body weight and inhibition in growth in animals receiving excess methionine has been reported from a number of laboratories.³⁻⁵ Excess methionine could alter the need for other amino acids, for example, by increasing the demand for them in methylating reactions,^{5,6} and by promoting the formation of one type of tissue protein at the expense of others. The question is raised as to whether or not the addition of other amino acids to the diet would reduce the drain on tissue sources which excess methionine could exert. The following experiments on rats were designed to answer this question in part.

Methods. Nitrogen balance indexes were

determined using the diet and technics previously described for the dog.⁷ The diet contained 12% casein (dry weight basis). Rats were pair-fed with those fed excess methionine (4.8%) where food intake was markedly restricted. The experiments were continued for a period of 20 days with urine and fecal collections throughout the period. At the end of the period the animals were autopsied and the livers and kidneys were dried at 95° C to constant weight. The dry livers and kidneys were analyzed for nitrogen by the micro-Kjeldahl procedure and were also analyzed for total fat. The urine was analyzed for creatinine and creatine by the Folin method.⁸

Results. The effects of relatively large addition of *l*-arginine and/or *dl*-methionine, to casein, on the nitrogen balance index and on the excretion of creatinine in the rat are recorded in Table I.

The average nitrogen balance index for casein was 0.81. The creatinine excretion on the casein diet was 28.3 mg/day/kg body weight. The addition of *l*-arginine to casein decreased the nitrogen balance index to 0.73 so that this amount of *l*-arginine did not improve the pattern of amino acids in the diet. Addition of excess *dl*-methionine to casein decreased the nitrogen balance index markedly so that the animal was in negative nitrogen balance and increased the excretion of creatinine significantly (34.3 mg), changes which

* These studies were supported in part by the Navy Department, Office of Naval Research.

† Gerard Swope Fellow of the General Electric Company.

1 Brown, J. H., and Allison, J. B., Abst. 112th Meeting of the Am. Chem. Soc., 1947, 51c.

2 Brown, J. H., Ph.D. Thesis, Rutgers University, 1948, in preparation for publication.

3 Earle, D. P., Small, K., and Victor, J., *J. Exp. Med.*, 1942, **76**, 317.

4 Riesen, W. H., Schweigert, B. S., and Elvehjem, C. A., *Arch. Biochem.*, 1946, **10**, 387.

5 MacKittrick, D. S., *Arch. Biochem.*, 1947, **15**, 133.

6 Bloch, K., and Schoenheimer, R. J., *J. Biol. Chem.*, 1941, **138**, 67.

7 Allison, J. B., Anderson, J. A., and Seeley, R. D., *Ann. N. Y. Acad. Sci.*, 1946, **47**, 245.

8 Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

TABLE I.
Nitrogen Balance Index and Creatinine Excretion in Rats. Average Data Obtained on 14 Animals.

Nitrogen source	Nitrogen balance index	Creatinine, mg/day/kg B.W.	Wt loss, g
Casein 12%	0.81	28.3 ± 1.2*	17 ± 2.1
12% casein + 1.7% arginine	0.73	31.2 ± 2.3	25 ± 5.1
12% " + 4.8% methionine	0.50	34.3 ± 0.9	59 ± 5.4
12% " + 1.7% arginine + 4.8% methionine	0.70	36.5 ± 0.7	35 ± 3.6

$$* e = \sqrt{\frac{\sum d^2}{n(n-1)}}$$

TABLE II.
Effects of Feeding *dl*-Methionine and/or *l*-Arginine in 12% Casein Diet on Amount of Liver Nitrogen and Liver Fat in Rats. Average Data Obtained on 14 Animals.

Nitrogen source	g N/g liver*	Liver wt 100 g B.W.	g liver N/100 g B.W.	Liver* fat, g%
Control 12% casein	0.096 ± .002	3.24 ± .02	0.096 ± .002	19 ± 1
12% casein + 1.7% arginine	0.099 ± .005	3.03 ± .07	0.093 ± .002	18 ± 2
12% " + 4.8% methionine	0.112 ± .004	3.19 ± .06	0.110 ± .002	13 ± 1
12% " + 4.8% " + 1.7% arginine	0.106 ± .001	2.86 ± .07	0.103 ± .003	15 ± 1

* Dry basis.

TABLE III.
Effect of Feeding *dl*-Methionine and/or *l*-Arginine in a 12% Casein Diet on Weight of Kidney and on Total Kidney Nitrogen in Rats.

Nitrogen sources	H ₂ O %	Kidney wt, g/100 g B.W.	Total nitrogen, g/100 g B.W.
12% casein	74 ± 1.1	0.69 ± 0.02	0.021 ± 0.002
12% " + 1.7% arginine	76 ± 1.2	0.67 ± 0.02	0.019 ± 0.002
12% " + 4.8% methionine	76 ± 1.2	0.95 ± 0.04	0.027 ± 0.003
12% " + 1.7% arginine + 4.8% methionine	74 ± 1.1	0.79 ± 0.05	0.023 ± 0.002

are associated with loss in nitrogen stores. The addition of arginine to the casein-methionine mixture increased the nitrogen balance index (0.7) above that for *dl*-methionine and casein alone, the creatinine excretion remaining at a high level (36.5 mg/day/100 g body weight). The increase in excretion of creatinine associated with excess methionine and arginine may be the result of increased synthesis of creatine.⁶

The close correlation between nitrogen balance index and weight loss is illustrated also in Table I. These data demonstrate that the loss in weight was much higher in animals fed the excess methionine and that the presence of arginine with the excess *dl*-methionine prevented some of this loss.

The effects of feeding *dl*-methionine and/or *l*-arginine in the 12% casein diet on the

amount of nitrogen in the liver and on liver fat are recorded in Table II.

The addition of *l*-arginine had little effect on the nitrogen of the liver but *dl*-methionine increased it, an increase which took place in the presence of a reduction in retention of nitrogen and a loss in body weight. The marked lipotropic effect of methionine is also shown by the reduction in the per cent of liver fat. Indeed the body fat is generally reduced below that found in the control animals, a reduction which contributed to the loss in body weight.

The addition of *dl*-methionine to the casein diet increased the size of the kidney while *l*-arginine alone had no effect (See Table III). The addition of *l*-arginine plus *dl*-methionine tended to increase the size of the kidneys but the increase was by no means as marked

as with *dl*-methionine alone, arginine having an antagonistic effect to methionine.

Summary. When excess *l*-arginine is added to casein, the nitrogen balance index is reduced slightly. An excess of *dl*-methionine on the other hand reduces the nitrogen balance index markedly and the excretion of creatinine is increased. When both *l*-arginine and *dl*-methionine are added to casein the nitrogen balance index is not reduced as much as it is in the presence of casein plus methionine, but the creatinine excretion is increased. The animals receiving 12% casein and paired with the group receiving 12% casein plus

4.8% *dl*-methionine lose weight slightly over a 20-day period. Those receiving 12% casein plus 4.8% methionine lose weight markedly, a loss which is overcome in part by adding 1.7% *l*-arginine to the methionine diet. The decrease in weight in the presence of a large excess of methionine is associated with a loss in body nitrogen and fat. The addition of *dl*-methionine increases the amount of nitrogen in the liver and reduces the amount of fat. Feeding *dl*-methionine plus casein increases the size of the kidney, an effect which is antagonized by *l*-arginine.

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Influence of Protein Reserves on Nephrectomized and Renal Artery Ligated Dogs.*

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The contrasting symptomatology, speed of elevation of blood nonprotein nitrogen and survival period of animals, after bilateral ligation of the renal arteries and removal of the kidneys has been reported.¹

Ligation of the arteries results in earlier and more severe evidence of toxicity, more rapid rise in blood nonprotein nitrogen and is more rapidly fatal. It is further of interest that the intravenous injection of renal extracts after bilateral nephrectomy tends to reproduce the effects of ligation of the arteries. In these circumstances toxicity and elevation of blood nonprotein nitrogen develop earlier and the duration of life is materially shortened. This evidence of association of accelerated nitrogen catabolism and toxicity is

reinforced by the results of protein feeding to animals after removal of the kidneys² and also after substantial reduction of kidney function.^{2,3} It points to a probable pathological nitrogen metabolism with production of toxic intermediates that represent some part of the augmented blood nonprotein nitrogen. Two factors seem to be concerned in the more rapid manifestations of toxicity and the rise in blood nonprotein nitrogen. They involve on the one hand some non-excretory renal function not yet known and on the other availability of tissue protein. The latter can be reasonably controlled by diet; this hypothesis is the basis of the experiments that follow.

Materials and Methods. Two groups of animals were given protein poor and protein rich diets respectively for protracted periods prior to the ligation of the renal arteries or the removal of the kidneys.

The daily ration of the protein poor diet per kilogram dog consisted of: Farina 15.0 g, Karo 2.5 g, Cod Liver Oil 0.4 g, Fresh yeast

* Aided by a grant from the Commonwealth Fund.

† James Hudson Brown Memorial Junior Fellow.

¹ Winternitz, M. C., Mylon, E., Waters, L. L., and Katzenstein, R., *Yale J. Biol. and Med.*, 1940, 12, 623.

² Mylon, E., Smith, E. R., and Goldstein, P., *Am. J. Physiol.*, 1948, 153, 55.

³ Addis, T., *Tr. Assn. Am. Phys.*, 1940, 55, 223.

The composition of the salt mixture follows:

	g
Potassium chloride	30
Sodium chloride	40
Magnesium chloride	20
Di-sodium phosphate	100
Monocalcium phosphate	100
Calcium lactate	40
Ferric ammonium citrate	10

Another group of 13 dogs were allowed 75 g each day of freshly ground horse meat per kilo—approximately the same caloric value as that of the protein poor diet. After 14 days of such feeding the renal arteries of seven of these animals were ligated and the kidneys removed in the other 6.

All operative procedures were facilitated with nembutal anesthesia, 30 mg per kilo. The exposures of the kidneys were by bilateral flank incisions and care was taken in ligation of the arteries to avoid inclusion of vein or ureter. The nonprotein nitrogen of the blood was determined with the half-micro Kjeldahl method.

Results and Discussion. As can be seen from Table I and Fig. 1 and 2, the height of the nitrogen catabolism as well as the degree of toxic symptoms following either operative procedure, are greatly influenced by the diet fed prior to the operations. Table I demonstrates both elevated nitrogen catabolism and reduction of the survival period of meat fed animals (Group 1 and Group 3) as compared to the 2 groups of dogs conditioned by the protein poor diet (Group 2 and 4).

In addition, the figures obtained for the animals of Group 1 and 3 indicate anew the contrasting symptomatology between bilateral ligation of renal arteries and removal of the kidneys. It can be seen that the former procedure results in a more rapid rise in blood

TABLE I.
Average N.P.N.'s (in mg %) Following Bilateral Renal Artery Ligation.

Group	Diet	No. of dogs	Days											
			1	2	3	4	5	6	7	8	9			
1	meat	7	45.5	115	211	293*								
2	carbohydrate	6	27	61.9	92.1	130.9	181.9	209.9†	283†	363†	395†			
* Avg of 3 survivors.														
† Values of the 6th dog.														
Average N.P.N.'s (in mg%) Following Bilateral Nephrectomy.														
Group	Diet	No. of dogs	Days											
			1	2	3	4	5	6	7	8	9	10	11	12
3	meat	6	47.9	91.9	150.2	217.3	278	307*	310†					
4	carbohydrate	3	30	56.8	87.1	120.6	163	190.3	234.7	281.6†	220§	208	252	
* Avg of 2 survivors.														
§ Values of the third dog.														

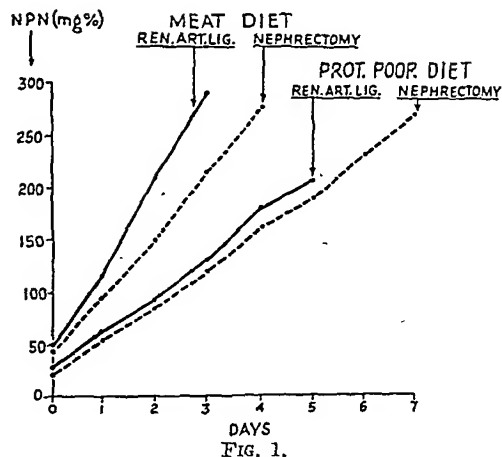


FIG. 1.

Nonprotein nitrogen of the blood of renal artery ligated and nephrectomized dogs pre-fed either a meat or a carbohydrate diet.

SURVIVAL TIME OF

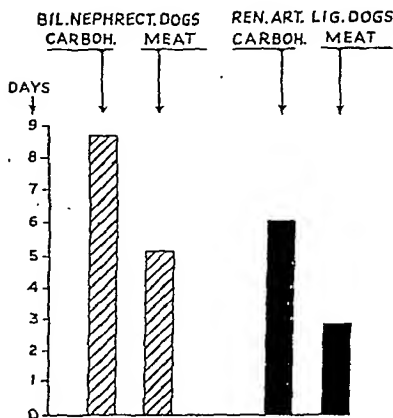


FIG. 2.

Survival time of renal artery ligated and nephrectomized dogs pre-fed either a meat or a carbohydrate diet.

nonprotein nitrogen[‡] and in a significant reduction in the survival time. While obviously these effects are caused by substances liberated from the anoxic kidney the mechanism of their action is not yet known. One lead may be available in the fact that these derivatives were only slightly active in animals of group 2, *i.e.* in those greatly deprived of their protein reserves. This observation suggests that renal substances, in themselves not very toxic, stimulate the intracellular nitrogen catabolism and that this stimulation is associated with the formation of toxic protein intermediates.

Summary and Conclusions. Renal artery ligation as well as nephrectomy was carried out in 2 groups of dogs, one maintained prior to the operation on a carbohydrate, the other on a protein diet. Following either operative procedure the nitrogen catabolism was more rapid, the toxic symptoms more severe, and the survival time shorter in protein pre-fed animals as compared with the carbohydrate group. In the former the contrasting results of renal artery ligation and nephrectomy are marked. It may be concluded both that substances derived from the ischemic kidney stimulate catabolism of protein reserves and that the toxic symptoms following renal artery ligation are associated with accelerated nitrogen catabolism.

[‡] The differences in the nonprotein nitrogen between animals of Groups 1 and 3 for 48 hours and 72 hours after the operative procedures are highly significant. The *p* values for these two time periods are <0.01 .

Antibacterial Activity of Gentisyl Alcohol and Homogentisic Acid.

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(Introduced by J. C. Forbes)

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It has been reported recently that gentisyl alcohol¹ and numerous derivatives of gentisyl alcohol^{2,3} have practically the same antibacterial activity as patulin* against *S. aureus* in plate tests. Raistrick and coworkers⁵ had reported previously that gentisyl alcohol had only slight activity against *S. aureus*, in contrast to the strong antibacterial activity of patulin.⁶

In preparation for studies involving homologous alcohols related to gentisyl alcohol we prepared gentisyl alcohol in order to test its antibacterial activity against *S. aureus*. In contrast to the reported findings of the Swiss workers,¹⁻³ but in agreement with the original report of Raistrick and coworkers,⁵ our results with gentisyl alcohol in the cylinder plate penicillin assay technic indicated only slight antibacterial activity over a broad pH range. For this reason work with the higher homologues of gentisyl alcohol is not contemplated at the present time, but in view of the relationship of homogentisic acid to the next higher homologue of gentisyl alcohol we have also tested this compound for antibacterial activity against *S. aureus*. We found it to have definite but slight antibacterial activity of about the same order of magnitude as that of gentisyl alcohol. Curves showing the anti-

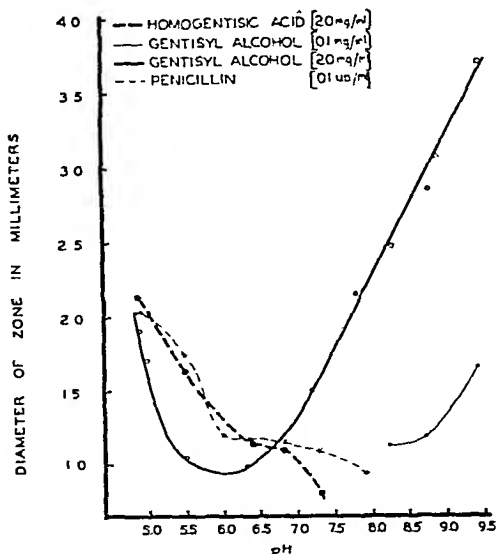


FIG. 1.

Effect of pH of the medium on the antibacterial activity of penicillin, gentisyl alcohol, and homogentisic acid.

bacterial action of gentisyl alcohol, homogentisic acid and penicillin in relation to pH of the medium are presented in Fig. 1.

Experimental. Gentisyl alcohol was prepared by catalytic hydrogenation of gentisaldehyde according to the method of Birkinshaw, Bracken and Raistrick.⁵ Synthetic homogentisic acid was prepared by a procedure which we intend to present in detail elsewhere. These compounds were purified by recrystallization several times and gave melting points agreeing with those reported in the literature.

Essentially the same cylinder plate procedure as that reported by Schmidt and Moyer⁷ for the assay of penicillin was used to de-

¹ Brack, A., *Helv. Chim. Acta*, 1947, 30, 1.

² Renz, J., *ibid.*, 1947, 30, 124.

³ Seebeck, E., *ibid.*, 1947, 30, 149.

* Also known as clavacin, clavatin, claviformin, and expansin.⁴

⁴ Waksman, S. A., *Microbial Antagonisms and Antibiotic Substances*, 2nd Ed., The Commonwealth Fund, New York, 1947, p. 210.

⁵ Birkinshaw, J. H., Bracken, A., and Raistrick, H., *Biochem. J.*, 1943, 37, 726.

⁶ Birkinshaw, J. H., Bracken, A., Michael, S. E., and Raistrick, H., *Lancet*, 1943, 2, 625.

⁷ Schmidt, W. H., and Moyer, A. J., *J. Bact.*, 1944, 47, 199.

termine antibacterial activity of the compounds. The only variation from this technique was in use of *Disco* "penassay" seed agar adjusted to variable hydrogen ion concentrations and the use of distilled water instead of buffer solutions to dissolve the penicillin and test compounds. Cylinders 8 mm in diameter were used, and *S. aureus* 209P was the test organism. All tests were run in quadruplicate, and an average of the 4 zone diameters was taken for the final reading.

Discussion. A concentration of 20 mg/ml of gentisyl alcohol or of homogentisic acid was necessary to produce antibacterial activity comparable to that shown by 0.1 unit/ml of the working standard of penicillin. Brack¹ reported antibacterial activity with concentrations of gentisyl alcohol of 100 γ /cc over the pH range 5.0 to 8.5. This degree of antibacterial activity was not observed in our experiments. We did note slight activity with this concentration of gentisyl alcohol in the pH range above 8.2. With 2% gentisyl alcohol antibacterial activity was more marked at low and at high pH values. Some oxidation of the compound occurs in the assay procedure, especially in alkaline media, and is evidenced by browning of the zones of inhibition. The possibility that oxidation products might have contributed to the antibacterial activity of

gentisyl alcohol noted in these cases has not been investigated. It will be noted that the pH activity curve usually observed with penicillin was obtained for control and comparative purposes in these experiments.

The various results of different laboratories might possibly be due to differences in media, test organism or to a combination of these two important factors, and they suggest that these factors may be critical in the evaluation of the antibacterial activity of gentisyl alcohol or its derivatives.

The slight antibacterial activity exhibited by homogentisic acid has not to our knowledge been described previously. This activity decreased rapidly with increase in the pH of the assay agar.

Summary. Gentisyl alcohol in our experiments was found to have only slight antibacterial activity over a broad pH range. Our results with this compound are at variance with those of Brack¹ but are in agreement with the original report of Raistrick and co-workers.⁵

Homogentisic acid was found to have a definite but slight antibacterial activity.

The effect of pH of the medium on the antibacterial activity of these compounds against *S. aureus* was studied.

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Purification of Psittacosis Virus by Alcohol Precipitation and Centrifugation.

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(Introduced by Keith H. Lewis)

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A number of procedures have been described in which chemical agents such as cal-

cium phosphate,¹ alum,² protamine,³ zinc and magnesium hydroxide⁴ are utilized in the concentration and partial purification of influenza

¹ Stanley, W. M., *Science*, 1945, **101**, 332.

² Bodily, H. L., Corey, M., and Eaton, M. D., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 165.

³ Chambers, L. A., and Henle, W., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 481.

virus. Recent experiments were reported by Cox *et al.*⁵ on the treatment of this virus with organic solvents such as methyl and ethyl alcohols and the possibility of applying the same procedure in the purification of other viruses was suggested. Accordingly, experiments were conducted to determine, (1) the effects of methyl and ethyl alcohol on the viability of psittacosis virus and (2) the purification value of this method compared to simple centrifugation.

Materials and Methods. The 6BC strain of virus used in these tests was originally obtained from Dr. K. F. Meyer. Following inoculation with an allantoic passage strain of this virus, the eggs were incubated at 37°C for 4 to 5 days. They were then candled and living embryos chilled at 4°C for 12 to 18 hours, after which the allantoic fluid was harvested aseptically and used as starting material. Usually the harvest was held another 18 to 24 hours at 4°C pending the results of sterility tests.

Infectivity titrations were performed by the single dilution method, *i.e.*, the inoculation of 0.25 ml of a single dilution of each test aliquot into the yolk sacs of 30 embryonated eggs. The eggs were candled at 24 hour intervals thereafter. The LD₅₀ is then calculated from the average day of death of the group of embryos.⁶

Total nitrogen determinations were made by the micro-Kjeldahl method.*

The alcohol precipitation method used was comparable to that recently described by Cox *et al.*⁵ To aliquots of infected allantoic fluid, cooled to 0°C, absolute methanol or ethanol (previously chilled to -40°C or below) was added dropwise through a capillary pipette until final concentrations of from 5% to 40% by volume were reached. The mixtures were

held at 0°C for approximately 3 hours and then centrifuged for 30 minutes at 3500 rpm in a No. PR-1 International Refrigerated centrifuge (0°C) equipped with an angle head rotor. The supernatant fluid was removed with a syringe and discarded. The precipitate was resuspended to original volume in 0.1 molar phosphate buffer, pH 7.4, and gently agitated on a mechanical shaker for 1 hour at room temperature. The suspension was then centrifuged at 1500 rpm for 15 minutes at room temperature in a No. 1 International centrifuge equipped with a horizontal rotor. The supernatant fluid, removed from the insoluble sediment, was titrated for virus activity in eggs by the method described. No pH adjustments were made.

Results. Following the completion of preliminary experiments on the behavior of psittacosis virus to the alcohol precipitation technic, tests were conducted to determine the effect of varying concentrations of methanol and ethanol on the viability of the virus.

Precipitation of allantoic fluid virus with varying concentrations of absolute methanol. To aliquots of infected allantoic fluid, previously centrifuged at 1500 rpm for 15 minutes to remove supposedly only large particles of extraneous material, absolute methanol (-40°C) was added in concentrations of from 5% to 40%, and the samples treated according to the procedure outlined. A control aliquot of virus from each starting virus suspension was held at 0°C during the period of the extraction. Upon completion of the process, each alcohol fraction was titrated along with the control for virus infectivity in embryonated eggs.

The results shown in Table I are typical of the observations made on several repeat experiments. There did not appear to be an optimum concentration of methanol between 5% and 30% which would consistently yield high recovery values of living virus as measured by infectivity tests. Although the results of tests made for mg total N/ml indicate that in most instances better than 90% of the nitrogen had been removed, the loss in infectivity titer varied from 0.2 to

⁴ McKinsty, D. W., and Cox, H. R., 1945, Unpublished experiments.

⁵ Cox, H. R., Van Der Scheer, J., Aiston, S., and Bohnel, E., *J. Immunol.*, 1947, **56**, 149.

⁶ Golub, O. J., *J. Immunol.*, 1948, in press.

*Determinations made by Dr. Benjamin Warshowsky of the Biological Division, Camp Detrick, Frederick, Md.

TABLE I.
Precipitation of Psittacosis Virus from Allantoic Fluid with Varying Concentrations of Methanol.

Lot No.	Type of data	Control	Precipitation by absolute methanol concentration of:						
			5%	10%	15%	20%	25%	30%	40%
1	LD ₅₀ (neg. log)	6.2	6.0	5.8	5.9				<2.0
	% recovery		63	40	50				<0.1
	Mg N/ml	0.516	0.017	0.018	0.019				0.057
2	LD ₅₀ (neg. log)	7.0			6.6	6.5	6.8	6.5	<2.0
	% recovery				40	32	63	32	<0.1
	Mg N/ml	0.447				0.021	0.027	0.035	0.042
3	LD ₅₀ (neg. log)	6.3			5.8	5.8	5.9	6.0	
	% recovery				32	32	40	50	
	Mg N/ml	0.504			0.034	0.050	0.055	0.049	

TABLE II.
Comparison of Virus Recoveries by Alcohol Precipitation at Different Temperatures.

Temp. of alcohol	% conc. of precipitant	Methanol			Ethanol		
		LD ₅₀ (neg. log)	% recovery	Mg N/ml	LD ₅₀ (neg. log)	% recovery	Mg N/ml
-40°C	0						
	(control)	6.4	*100	.588	6.4	100	.588
	10	6.0	40	.012	6.0	40	.024
	25	6.5	100	.054	<2.0	<.1	.066
	40	<2.0	<.1	.090	<2.0	<.1	.064
0°C	0						
	(control)	6.4	100	.588	6.4	100	.588
	10	5.5	13	.050	5.6	16	.021
	25	6.0	40	.045	<2.0	<.1	.064
	40	<2.0	<.1	.070	<2.0	<.1	.051

* The control LD₅₀ is given a value of 100%. The per cent recoveries of precipitated suspensions are all based on this figure. The control for both the ethanol and methanol series is the same virus suspension.

0.5 log, indicating recoveries between 63 and 32%, respectively, of the original virus material. Methanol concentrations of 40% total volume were in all cases definitely destructive to the virus.

Precipitation of allantoic fluid with absolute ethanol as compared to methanol (at -40°C and 0°C). In view of the poor recovery values obtained with methanol (-40°C), it was decided to make comparative tests using absolute ethanol as the precipitant at both -40°C and 0°C. To measured aliquots of the virus chilled to 0°C were added varying concentrations of the alcohol previously chilled to either (1) below -40°C in an acetone-dry ice bath, or (2) 0°C in an ice-water bath. The results are shown in Table II.

It can be seen that when the precipitations were performed at either temperature, a concentration of 25% ethanol was toxic, whereas fair or good recoveries were obtainable with methanol at the same concentration. At the 40% level, both alcohols resulted in a marked inactivation of the virus at either temperature. The probable greater denaturation of virus protein resulting from ethanol activity is in agreement with similar observations made by Liu *et al.*⁷ on denaturation of serum proteins. The results of total nitrogen determinations indicated that similar purifications resulted whether ethanol or methanol was used.

⁷ Liu, Szu-Chih, and Wu, Hsien, *Chinese J. Physiol.*, 1937, 11, 315.

In view of the observations made by Lazarus⁸ on the comparative susceptibility of psittacosis virus to trypsin, attempts were made to digest the proteins of infected allantoic fluid by preliminary treatment with this enzyme. Although the virus withstood the action of the trypsin, it was not possible to demonstrate any increased purification when the digestion was done prior to alcohol precipitation.

In the course of our studies on the mechanism of the precipitation it became increasingly evident that, at least in the case of psittacosis virus, the sedimentation of the virus during the alcohol procedure was in all probability due to centrifugation (3500 rpm/30 minutes) and not to a virus protein precipitation. It was apparent also that preliminary centrifugation of infected allantoic fluids at 1500 rpm for 15 minutes was sedimenting not only large particulate matter, but a considerable proportion of the virus as well. This was especially true when the harvested allantoic fluid contained large quantities of insoluble material. Aliquots of original infected fluid, not subjected to any preliminary centrifugation, were precipitated with absolute methanol at -76°C and centrifuged at varying speeds from 1500 to 3500 rpm for 30 minutes, after which the sediments were suspended in phosphate buffer and tested for virus activity as previously described. A second virus preparation, previously purified by centrifugation,[†] was also subjected to the same alcohol procedure. Aliquots of the same

TABLE III.
Comparison of Virus Recoveries by Centrifugation and by Alcohol Precipitation.

Virus	Speed of centrifugation (rpm)	Alcohol precipitation*			Centrifugation control			Uncentrifuged refrigerated control	
		LD ₅₀ (neg. log)	% recovery	Mg N/ml	LD ₅₀ (neg. log)	% recovery	Mg N/ml	LD ₅₀ (neg. log)	Mg N/ml
Original allantoic fluid	1500	7.1	100	.053	7.0	100	.050		
	2500	7.0	100	.052	7.5	100	.034		
	3500	7.1	100	.052	7.4	100	.032	7.0	.548
Partially purified allantoic fluid	1500	6.1	13	.017	6.1	14	.012		
	2500	6.6	40	.011	6.7	50	.011		
	3500	6.9	80	.012	6.8	63	.013	7.0	.016

* All aliquots were precipitated with 25% by volume of absolute methanol.

⁸ Lazarus, A. S., and Meyer, K. F., *J. Bact.*, 1939, **38**, 121.

[†] Preliminary purification was accomplished as follows: original infected allantoic fluid was spun at 4000 r.p.m. for 60 minutes in a refrigerated angle-head centrifuge. The supernatant fluid was discarded and the sediment resuspended to approximately one-half the original volume in phosphate buffer, shaken with glass beads for several minutes, and centrifuged at 2000 r.p.m. for 15 minutes. The supernatant fluid was removed, spun again at 4000 r.p.m. for 60 minutes and the sediment resuspended to the original volume in buffer solution. This procedure removes most of the extraneous protein and cell debris without significant loss of active virus.

parent pools, not treated with alcohol, were included as controls.

Table III shows the results obtained in terms of the virus recovery and N content. It is apparent that when such preparations of original allantoic fluid are subjected to centrifugation alone, at speeds even as low as 1500 rpm, the virus is sedimented and recoverable in its entirety, within the limits of the error of the methods employed. Several virus samples indicated greater than 100% recovery, and it is believed that these are the results of disaggregation of virus clumps normally present in the original allantoic fluid. When a partially purified virus suspension was employed, the centrifugal force necessary to bring down virus activity was definitely increased but the addition of alcohol did not augment the recovery of virus. The N content of the partially purified virus suspension (Table III, under Refrigerated Control) is further shown to be lower than in the original allantoic fluid preparations treated with alcohol. The original fluid, which was subjected only to varying speeds

of centrifugation from 1500 to 3500 rpm for 30 minutes, demonstrates as great a reduction in non-viral N as those preparations undergoing alcohol precipitation. Therefore, it must be concluded that the alcohol did not act as a virus precipitant with this agent and that the primary factor in sedimentation and purification by this procedure was the simple centrifugation employed in the process.

Summary. Experiments are reported on the effect of methanol and ethanol on the viability and purification of psittacosis virus. A comparison of this technic with differential centrifugation is presented.

The results suggest that the separation of virus from crude allantoic fluid was dependent on a non-specific adsorption of the virus on particulate matter either present originally in the allantoic fluid or brought out of solution by the alcohol. With virus suspensions first purified by centrifugation, the speed of centrifugation and not the precipitation by alcohol was the determining factor in the recovery of virus.

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Changes in Ascorbic Acid Metabolism of the Rat During Infection with *Trypanosoma hippicum*.*

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Investigation of the biochemical changes occurring in the host during trypanosomiasis has been directed primarily toward the observation of changes in carbohydrate metabolism.¹ There have been some studies, however, which indicate that the ascorbic acid

metabolism of the host is affected during infection. Scheff and Csillag² showed that the ascorbic acid content of the liver and blood of guinea pigs is lowered during infection with *Trypanosoma brucei*. Ascorbic acid administration was reported by Perla³ to increase the resistance of guinea pigs to infection with *T. brucei*, but not to alter the course of the infection in mice. These findings sup-

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¹ VonBrand, T., *Quart. Rev. Biol.*, 1938, 13, 41.

² Scheff, G., and Csillag, Z., *Arch. f. exp. Path. Pharmacol.*, 1938, 183, 467.

³ Perla, D., *Am. J. Hyg.*, 1937, 26, 374.

ported the report made by Borghi⁴ that scorbutic guinea pigs showed an increased susceptibility to infection with *T. brucei*.

The present study was undertaken to investigate alterations in the content of the tissue ascorbic acid of rats infected with *T. hippicum*. The distribution of oxidized and reduced ascorbic acid was studied in the hope that analysis for both forms would yield some information leading to a better understanding of the biochemical changes observed during trypanosomiasis.

Methods and Materials. Male Sprague-Dawley rats weighing 200-300 g were kept on a uniform diet⁵ 3 weeks previous to use in order to attain a constant level of ascorbic acid in the tissues. The animals were divided into two groups. The first, or control, group was sacrificed at the end of the 3 weeks feeding period, while the second group was infected after this feeding period and then maintained on the same diet during the course of infection. Both normal and infected animals were fasted 24 hours before their tissues were taken for analysis. The strain of *T. hippicum* used⁴ was maintained by passage through rats, which were infected by intraperitoneal injection of the trypanosomes suspended in Ringer-Locke solution at pH 7.4. About 36 hours after infection the level of the parasitemia was determined by hemocytometer. From this information it was possible to predict the time of death to within 6 hours of its occurrence which was, on the average, 60 hours after infection. The infected rats were sacrificed after pre-mortal symptoms appeared, but before the terminal convulsions occurred.

Samples of liver, spleen, adrenal, skeletal muscle and plasma were taken for analysis. In the assay of the adrenals, one gland was used for the determination of oxidized ascorbic acid and the other for total ascorbic

acid. Plasma, free of trypanosomes, was obtained by differential centrifugation of heparinized blood collected after decapitation of the animals. The tissues were homogenized⁶ in a 5% trichloroacetic acid for total ascorbic acid, or in 6% metaphosphoric acid containing 0.5% thiourea for dehydroascorbic acid analyses. Samples of plasma were added to similar acid solutions. Dehydroascorbic acid was measured in the acid extracts by the method of Roe and Oesterling⁷ modified by Herrmann and DuBois,⁸ and total ascorbic acid was measured by the method of Bessey, Lowry, and Brock.⁹ The spectrophotometric measurements were carried out on a Coleman spectrophotometer at 520 mμ wave length. Values for reduced ascorbic acid were calculated by subtracting the values for dehydroascorbic acid from those of the total.

All values for tissue ascorbic acid were expressed as mg of ascorbic acid per g of dry tissue. There was no significant difference between the water content of the tissues of the infected rats and that of the control rats. The results of the plasma analyses were expressed as mg of ascorbic acid per g of plasma. Means were calculated for both the control and infected animals, and the significance of the results was evaluated by calculating the "t" values between the means of the experimental and control data, in which

$$t = \frac{m_1^2 - m_2^2}{\sqrt{e_1^2 + e_2^2}} \cdot 10$$

Results. The figures in part 1 of the table show that a large decrease was observed in the total ascorbic acid content of the spleen. The greatest proportionate decrease took place in the reduced fraction, while the dehydroascorbic acid showed a slight, but not very significant, decrease in the infected animals. In the course of these experiments it was

⁶ Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

⁷ Roe, J. H., and Oesterling, M. J., *J. Biol. Chem.*, 1944, **152**, 511.

⁸ Herrmann, R., and DuBois, K., unpublished.

⁹ Bessey, O., Lowry, O., and Brock, M. J., *J. Biol. Chem.*, 1947, **168**, 107.

¹⁰ Burn, J. H., Biological Standardization, Oxford University Press, London, 1937, 29.

⁴ Borghi, B., *Atti accad. Lincei*, 1933, **17**, 665.

⁵ Hasch, Z., and Hajdu, I., *Pflügers Arch.*, 1939, **241**, 507.

[†] The strain of *T. hippicum* used in these experiments was kindly supplied by Dr. M. H. Soule, University of Michigan.

observed that the ratio of the wet weight of the spleen to the total body weight increased from 1.94×10^{-3} in the controls to 5.46×10^{-3} in the infected rats, an increase of 281% in the spleen to body weight ratio. When the total amount of ascorbic acid was calculated from the weight of the total spleen and the concentration of ascorbic acid in mg per g of wet tissue, it was found to increase only 175%. This indicated that the decrease observed in the spleen was an actual diminution in the ascorbic acid content, and was not due to a dilution of the ascorbic acid normally present as the result of splenic hypertrophy.

The results of the ascorbic acid analyses on the adrenals are presented in part II of the table. The total ascorbic acid of the adrenals of the infected animals decreased significantly. The primary alteration occurred in the reduced fraction, while the dehydroascorbic acid showed no change from normal. Since the ascorbic acid of the adrenal is concentrated in the cortex, the change represented by these results would be more pronounced if the determination were based on the cortex alone, rather than upon the entire adrenal.

The changes which took place in the liver ascorbic acid are shown in part III of the table. The total ascorbic acid decreased during infection. As in the spleen and adrenal, the greatest proportionate decrease occurred in the reduced form. The dehydroascorbic acid of the liver of the infected animals was also less than that observed in the controls, but this decrease was not statistically significant.

The ascorbic acid concentration of the muscle was not greatly affected by infection with *T. hippicum*. It can be seen from part IV of the table that, although the muscle dehydroascorbic acid increased slightly, the significance of the increase ($t = 2.9$) is open to question.

The figures in part V of the table show that the ascorbic acid of the plasma was elevated above normal. Unlike the tissues discussed previously, both fractions were increased to the same extent. The dehydroascorbic and reduced ascorbic acid each in-

creased 100% in the plasma of the infected rats.

Discussion. The lowered concentration of reduced ascorbic acid in the spleen and adrenal, without an equivalent decrease in oxidized ascorbic acid, indicates that there was an interference in the oxidation-reduction processes of the host during infection, which may have been responsible for the changes observed in the distribution of ascorbic acid in the infected animals. These findings are comparable to those of Scheff and Csillag¹² who found that the reduced glutathione decreased while the total glutathione did not change in the blood and liver of guinea pigs infected with *T. brucei*. To a lesser extent, a similar decrease took place in the ratio of reduced to oxidized ascorbic acid in the muscle, because of the increase observed in dehydroascorbic acid. In the liver and plasma, the normal ratio was not measurably altered.

The decrease in ascorbic acid in all tissues studied, except muscle, accompanied by an increase in plasma ascorbic acid, suggested that the metabolite may have diffused from the tissues to the blood. This explanation does not seem wholly adequate, since a greater decrease took place in the reduced fraction in the depleted tissues, while both forms of ascorbic acid increased in the plasma.

Our knowledge of the metabolic requirements of trypanosomes is limited, but it is of interest to note that Groat and Mora¹¹ found that their strain of *T. cruzi* required reduced ascorbic acid for cultivation *in vitro*. Further investigation of the changes produced in the oxidation-reduction systems of the host by *T. hippicum* may reveal the significance of the alterations in the distribution of ascorbic acid observed and their relation to the symptomatology of the infection.

Summary. Dehydroascorbic and total ascorbic acid were measured in the liver, spleen, adrenals, muscle, and plasma of rats infected with *T. hippicum* and the reduced ascorbic acid content of these tissues was calculated.

¹¹ Groat, H., and Mora, C., *Annals soc. biol. Bogota*, 1947, 2, 188.

TABLE I. The Effect of *T. hippicum* Infection on Ascorbic Acid Content of Rat Tissues. Ascorbic acid content is expressed as mg per g of dry tissue. In the spleen, adrenal, liver and muscle, 7 control animals and 7 infected animals were used. In the analyses made upon the plasma, 2 separate groups of 8 animals each were used.

	Animal No.	Total mg/g		Dehydro. mg/g		Reduced mg/g (Total-dehydro.)	
		Control	Infected	Control	Infected	Control	Infected
Spleen I	1	3.22	1.75	0.59	0.34	2.63	1.41
	2	3.04	2.14	0.46	0.27	2.58	1.87
	3	2.83	1.64	0.20	0.34	2.63	1.30
	4	3.14	1.72	0.35	0.19	2.79	1.53
	5	3.00	1.72	0.30	0.23	2.70	1.49
	6	2.79	1.96	0.36	0.26	2.43	1.70
	7	2.92	2.07	0.33	0.26	2.59	1.81
	mean	2.99	1.86	0.37	0.27	2.62	1.59
	ϵ	0.06	0.07	0.05	0.02	0.06	0.08
	t^*	11.9		2.0		10.3	
Adrenal II	1	12.75	11.41	0.99	1.03	11.76	10.38
	2	14.96	8.77	1.24	0.52	13.72	8.25
	3	—	11.65	1.26	1.39	—	10.25
	4	12.67	9.02	0.83	0.98	11.84	8.04
	5	13.50	8.31	1.02	0.82	12.48	7.49
	6	10.98	10.25	0.91	1.17	10.07	9.08
	7	13.74	8.77	0.71	0.74	13.03	8.03
	mean	13.10	9.70	0.99	0.95	12.15	8.79
	ϵ	0.54	0.51	0.08	0.11	0.52	0.43
	t	4.6		0.31		5.0	
Liver III	1	1.22	1.01	0.29	0.32	0.93	0.79
	2	1.38	0.66	0.23	0.09	1.12	0.57
	3	1.28	1.03	0.35	0.23	0.93	0.80
	4	1.10	0.84	0.12	0.08	0.98	0.76
	5	1.59	0.80	0.15	0.08	1.43	0.72
	6	1.08	0.78	0.17	0.11	0.91	0.67
	7	1.17	0.67	0.13	0.13	1.04	0.54
	mean	1.26	0.83	0.21	0.15	1.05	0.69
	ϵ	0.07	0.06	0.03	0.03	0.07	0.04
	t	4.90		1.4		4.5	
Muscle IV	1	0.18	0.23	0.06	0.16	0.12	0.07
	2	0.17	0.33	0.08	0.10	0.09	0.23
	3	0.20	0.20	0.07	0.14	0.12	0.07
	4	0.25	0.20	0.07	0.06	0.18	0.14
	5	0.20	0.24	0.07	0.06	0.12	0.17
	6	0.20	0.20	0.07	0.09	0.12	0.11
	7	0.18	0.20	0.07	0.09	0.11	0.11
	mean	0.20	0.23	0.07	0.10	0.12	0.13
	ϵ	0.01	0.02	0.003	0.01	0.01	0.02
	t	4.90		1.4		4.5	
Plasma V†	1	0.015	0.041	0.010	0.017	0.005	0.024
	2	0.025	0.042	0.016	0.032	0.009	0.010
	3	0.017	0.050	0.012	—	0.005	—
	4	0.023	0.046	0.009	0.028	0.014	0.018
	5	0.019	0.042	0.014	0.033	0.005	0.009
	6	0.026	0.050	0.015	0.039	0.011	0.011
	7	0.016	0.051	0.008	0.021	0.008	0.030
	8	0.020	0.032	0.017	0.020	0.003	0.012
	mean	0.020	0.044	0.013	0.027	0.008	0.016
	ϵ	0.002	0.003	0.001	0.003	0.001	0.003
	t	9.6		4.4		2.7	

$$*t = \frac{m_1 - m_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}}$$

† mg ascorbic acid/g of plasma.

The liver, spleen and adrenals showed significant decreases in reduced ascorbic acid content. The dehydroascorbic acid content of

the muscle was increased slightly and both dehydroascorbic and reduced ascorbic acid of the plasma were increased two-fold.

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Effect of Environmental Temperature Upon Clotting Time of Whole Blood.

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The *in vitro* clotting time of normal whole blood, as reported by various workers, has a wide range of activity, varying from one minute to over 15 minutes.¹ There are several factors responsible for the wide variations. One important consideration is the method of obtaining blood samples. When finger puncture methods are used, there is a variable degree of contamination with thromboplastic tissue substances. With venous blood, the tissue substances are important only when venipuncture is not smooth. Another major variable is the degree of trauma and surface contact to which the blood is subjected during the determination. When the venipuncture is clean, and the apparatus and manipulations are standardized so that traumatic factors are kept constant, the only remaining significant extrinsic variable is the temperature. It is the purpose of this paper to indicate the magnitude of the effect of room temperature on the clotting time of whole blood.

In order to standardize the trauma factor and retain a precise endpoint, a rotating tube method for clotting time has been developed.¹ By this method 0.5 ml of venous blood is promptly placed in the bulbous end of a tube rotated around its long axis at a slow constant rate (one r.p.m.) by an electric motor. When the blood clots, the fibrous

strands adhere to the ascending wall of the tube and are thus readily detected. The advantages of this method are that the degree of trauma and surface contact is constant and mechanically determined, and the endpoint is precise.

In order to determine the influence of temperature on the clotting time by this method, three identical sets of apparatus were set up at three different temperatures. One-half ml aliquots of the same samples of blood were delivered into bulbous tubes and the clotting time (fibrin appearance time) determined at the 3 different temperatures. The

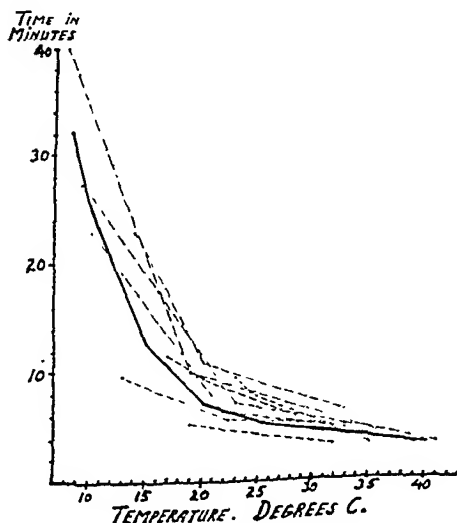


FIG. 1.

Relationship of clotting time to room temperature.

¹ Weiner, M., and Shapiro, S., *J. Lab. and Clin. Med.*, 1947, 32, 1037.

blood samples were taken from patients chosen at random. In Fig. 1 the temperatures at which each clotting time was determined is plotted against the corresponding clotting time, and points determined with the same sample of blood are connected by broken lines. The solid line represents the estimated average slope of these broken lines. Its position in the figure is based on the average normal value of 5 min. 28 sec. previously determined at about 26°C. At this temperature, 4 to 6 3/4 min. is considered the normal range. It is obvious from the curve that winter-summer variations of room temperature, which may range from 19°C

to 37°C in temperate climates, can result in considerable changes in the normal range of clotting time.

It is recommended that a curve such as shown in Fig. 1 be used to evaluate clotting time results whenever this determination is performed at room temperature.

Summary. The effect of room temperature on the clotting time of whole blood determined by the rotating tube method is described. The clotting time may be significantly prolonged in cool environments, and shortened in warm weather. A curve is described to correct for this factor.

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Pteroylglutamic Acid Deficiency in Mice: Hematologic and Histologic Findings.*

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The production of a deficiency of pteroylglutamic acid (PGA) can be accomplished in various mammalian species by feeding a purified diet containing the known essential vitamins, excluding PGA, to which has been added either succinylsulfathiazole, a PGA antagonist, or both. In the deficient state the animals have shown varying degrees of reduction of all circulating elements in the blood stream. Reports of bone marrow findings have been conflicting and incomplete and have not been reported previously for mice.

Procedure. Deficiency may be established in weanling mice more quickly than in adults. Weanling animals of a Swiss strain with an average weight of about 20 g were placed in wire mesh cages and were allowed distilled

water ad libitum. They were fed unlimited quantities of a PGA free diet of the following percentage composition:

Glucose ("Cerelose")	59.1
Casein ("Labco" vitamin free)	20.0
Cellulose ("Cellu-flo" or "Ruffex")	5.0
Salts USP (Smaco)	3.8
Accessory salts*	0.2
Choline chloride	0.2
Inositol	0.1
Thiamine hydrochloride	0.0005
Riboflavin	0.001
Pyridoxine hydrochloride	0.0005
Niacinamide	0.005
Calcium pantothenate	0.0055
Ascorbic acid	0.01
2-methyl-1, 4-naphthoquinone	0.001
Biotin	0.00001
Hydrogenated vegetable oils ("Primex")	8.0
Corn oil containing vit. A, D, and E†	2.0

* Accessory salts contained in 0.2 g (expressed in mg): KCl, 100; NaCl, 87.2; FeSO₄, 10; MnSO₄, 1; ZnSO₄·7H₂O, 1; CuSO₄·5H₂O, 1; NaI, 0.3; NaF, 0.1; CoCl₂·6H₂O, 0.01.

† Two grams of the corn oil preparation contained vitamin A, about 4500 units; vitamin D, about 560 units; and mixed tocopherols, about 2.7 mg.

* This research was made possible by a grant from the Price McKinney Memorial Fund and is part of a study aided by a grant from the United States Public Health Service.

The liver, spleen and adrenals showed significant decreases in reduced ascorbic acid content. The dehydroascorbic acid content of

the muscle was increased slightly and both dehydroascorbic and reduced ascorbic acid of the plasma were increased two-fold.

16671

Effect of Environmental Temperature Upon Clotting Time of Whole Blood.

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The *in vitro* clotting time of normal whole blood, as reported by various workers, has a wide range of activity, varying from one minute to over 15 minutes.¹ There are several factors responsible for the wide variations. One important consideration is the method of obtaining blood samples. When finger puncture methods are used, there is a variable degree of contamination with thromboplastic tissue substances. With venous blood, the tissue substances are important only when venipuncture is not smooth. Another major variable is the degree of trauma and surface contact to which the blood is subjected during the determination. When the venipuncture is clean, and the apparatus and manipulations are standardized so that traumatic factors are kept constant, the only remaining significant extrinsic variable is the temperature. It is the purpose of this paper to indicate the magnitude of the effect of room temperature on the clotting time of whole blood.

In order to standardize the trauma factor and retain a precise endpoint, a rotating tube method for clotting time has been developed.¹ By this method 0.5 ml of venous blood is promptly placed in the bulbous end of a tube rotated around its long axis at a slow constant rate (one r.p.m.) by an electric motor. When the blood clots, the fibrous

strands adhere to the ascending wall of the tube and are thus readily detected. The advantages of this method are that the degree of trauma and surface contact is constant and mechanically determined, and the endpoint is precise.

In order to determine the influence of temperature on the clotting time by this method, three identical sets of apparatus were set up at three different temperatures. One-half ml aliquots of the same samples of blood were delivered into bulbous tubes and the clotting time (fibrin appearance time) determined at the 3 different temperatures. The

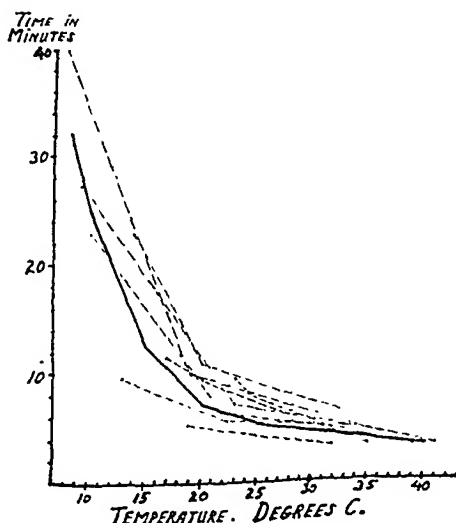


FIG. 1.

Relationship of clotting time to room temperature.

¹ Weiner, M., and Shapiro, S., *J. Lab. and Clin. Med.*, 1947, 32, 1037.

PLATE 3. Normal Marrow. $\times 410$. Note the presence of numerous nucleated red blood cells, polymorphonuclear leucocytes, and megakaryocytes. The absence of fat is normal in the mouse.

PLATE 4. Deficient Marrow. $\times 410$. Large immature cells with nucleoli and mitoses predominate. Almost no polymorphonuclear leucocytes or nucleated red cells are seen and megakaryocytes have disappeared.

Five-tenths percent of a crude antagonist of PGA (Lederle N-67A)^{1†} was added to the diet. In a preliminary experiment succinyl-sulfathiazole[‡] also was added but it was found that the majority of animals died before the desired evidences of deficiency had developed. Therefore the sulfonamide was withheld for the first month and then added at a level of 1.0% in the diet. With this modification only a very small percentage of the animals died in the preliminary stages of the experiment. On this regimen, deficiency was established in all animals within 50 to 60 days. A number then were killed for histologic studies. The remainder of the group was divided into 3 sub-groups and given varying amounts of PGA in the form of solutions of 'Folvite' (Lederle). Daily subcutaneous injections of 20, 2, and 0.2 μg were used. Care was taken to protect the solutions from exposure to sources of ultra-violet radiation. At weekly intervals the animals were weighed and white blood cell counts were done. Normally leucocyte counts of mice are not less than 6000 per cmm. When levels below 4000 were reached, it was considered that PGA deficiency was established. Red blood cell counts were then done; and, during the period of treatment with PGA, daily reticulocyte counts also were performed.

Hematologic Findings. After 30 to 40 days of the above regimen the white blood cell counts begin to fall to low normal levels. By 50 days many counts are below the minimal normal level of 6000, and by 60 days

practically all the counts are below 4000 with some as low as 1000. Both lymphocytes and granulocytes participate in this leucopenia. There is no consistent change in the differential count; the normal lymphocyte-to-granulocyte ratio is preserved. Red blood cell counts done at the time when the leucocytes had fallen to levels of less than 6000 per cc showed that a moderate to marked anemia had developed. From a normal count of about 10 million erythrocytes per cc the count was usually reduced to 4 to 6 million with some counts as low as 2 million per cc.

The entire blood picture may be restored rapidly to normal by the parenteral administration of PGA while the animals are still on the dietary regimen with the added antagonist and succinylsulfathiazole. A maximal response was obtained by giving daily subcutaneous injections of 20 μg of a solution of 'Folvite' (Lederle). There is a reticulocyte rise in 2 days which usually reaches a maximum of about 30% in 7 days. The erythrocyte counts become normal within 15 to 20 days. Also there is a very rapid regeneration of leucocytes, normal counts being obtained within 3 to 5 days. More remarkable is the fact that abnormally high white counts may occur. In one animal the initial leucocyte count of 2000 per cc rose to 57,000 per cc 3 days after PGA was started, and to 85,000 per cc 3 days later; after another 3 days it was below the normal maximum of 25,000 per cc.

During the first month prior to the administration of succinylsulfathiazole there is a moderate gain in weight. On the addition of this drug to the purified diet the mice soon cease to gain in weight and many animals show losses of a few grams. When PGA is given, weight gain is resumed at a rapid rate.

¹ Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., *J. Biol. Chem.*, 1947, **169**, 427.

[†] Generously supplied by the Lederle Laboratories Division of the American Cyanamid Co.

[‡] Kindly furnished by the Medical Research Division of Sharp and Dohme, Inc.

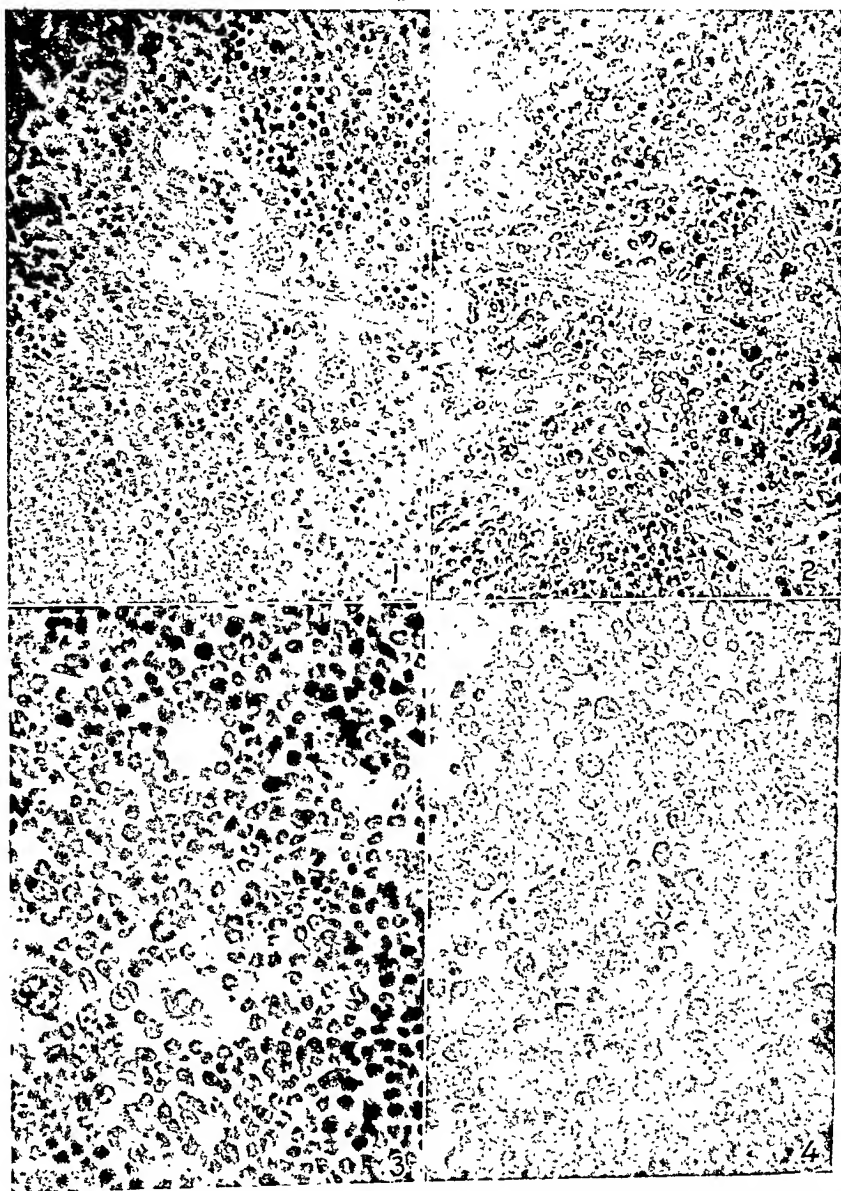


PLATE 1. Normal Spleen. $\times 246$. Note the degree of cellularity and the numerous nucleated red blood cells and megakaryocytes. Actually this spleen is from a mouse that had been deficient and was cured by the administration of PGA.

PLATE 2. Deficient Spleen. $\times 246$. The cellularity is much reduced. Nucleated red blood cells and megakaryocytes have practically disappeared. Extensive deposits of hemosiderin are present but these cannot be appreciated in the absence of color-reproduction. The coarsely granular appearance of some of the cells is due to hemosiderin.

ing peripheral panhematopenia; detailed studies of the marrow were not included in the report. Heinle, Welch, George, Epstein, and Pritchard,⁶ using swine, found megaloblastic hyperplasia of the marrow. Franklin, Stokstad, Belt, and Jukes,¹ using rats, observed an increase of nucleated red blood cells in the marrow, an increased number of blast cells, and a marked reduction of the polymorphonuclear leucocytes. They stated that the maturation of cells of the erythroid series and the production of mature granulocytes were seriously impaired. They did not stress hyperplasia with maturation arrest. Cartwright, Fay, Tatting, and Wintrobe,⁷ using swine, found a reduction of polymorphonuclear leucocytes and metamyelocytes, a slight increase in earlier forms of the myeloid series, and a significant decrease in the leucocyte-erythrocyte ratio. They observed immature and nucleated red cells which they considered to be similar in many ways to those seen in the marrow of patients with pernicious anemia in relapse.

The exact role of PGA in the formation and maturation of blood cells has not been elucidated. Certainly it participates in an important way. If it were an essential growth factor necessary for the formation of the most

primitive blood cells we would expect pancytopenia of bone marrow, rather than maturation arrest and hyperplasia of immature elements. It may be inferred, then, that PGA does not participate in the production of the most primitive blood elements, but rather that it must be present to allow maturation beyond the most primitive stages. In pernicious anemia in relapse, and in some cases of agranulocytosis, a similar although an apparently selective maturation arrest with hyperplasia of immature elements is seen. The production of primitive elements is not abolished and may even be in excess. It has been postulated that in red cell formation the role of the anti-pernicious anemia factor of liver is to act in conjunction with PGA or its conjugates to bring about the maturation of red blood cells beyond the megaloblastic stage. This would fit into our hypothesis that PGA may not be necessary for the formation of the most primitive blood elements, but that it is concerned with reactions essential to maturation such as loss of nuclear material.

Summary. 1. A deficiency of pteroylglutamic acid in mice produces a reduction of all cellular elements of the circulating blood.

2. In the deficient state, the bone marrow of mice shows maturation arrest with hyperplasia of immature elements and marked reduction of more adult forms.

3. It is suggested that pteroylglutamic acid may not be necessary for the formation of the most immature blood elements, but must be present to allow maturation after these primitive elements are formed.

⁶ Heinle, R. W., Welch, A. D., George, W. L., Epstein, M., and Pritchard, J. A., *Proc. Central Soc. Clin. Research*, 1947, **20**, 7.

⁷ Cartwright, G. E., Fay, J., Tatting, B., and Wintrobe, M. W., *J. Lab. and Clin. Med.*, 1948, **33**, 397.

Also the ruffled fur characteristic of the deficient state disappears.

Histologic Findings. See Plates 1-4. The spleen of normal mice is an active hematopoietic organ. Many megakaryocytes are present and numerous foci of nucleated red blood cells are scattered diffusely through the pulp. There are a few polymorphonuclear leucocytes and occasionally immature cells. Mitoses are rarely seen. In PGA deficiency the hematopoietic activity of the spleen practically ceases. Megakaryocytes and nucleated red cells may entirely disappear. Polymorphonuclear leucocytes are decreased in number. The pulp becomes sparsely cellular and shows large amounts of hemosiderin, both in the free form and phagocytized by macrophages. As in pernicious anemia, the hemosiderin probably represents iron released by the normal breakdown of erythrocytes. In PGA deficiency it is not utilized because of the decreased formation of erythrocytes. The splenic follicles did not show any consistent change from the normal. Treatment with PGA restores the histology of the spleen to normal, although some of the hemosiderin may persist. In a few animals, in the deficient state, there was a moderate increase in the number of immature cells in the splenic pulp in addition to the findings described above. This was never as prominent in the spleen as in the bone marrow.

The marrow of normal mice contains practically no fat. The cellular elements consist of nucleated red blood cells, megakaryocytes, polymorphonuclear leucocytes, a few immature cells, a few lymphocytes, and rare mitoses. In PGA deficiency a very marked change occurs. The marrow becomes solidly packed with large immature cells containing nucleoli and showing numerous mitoses. Only a few identifiable nucleated red cells remain. Polymorphonuclear leucocytes and megakaryocytes may disappear entirely. The picture is one of maturation arrest with overproduction of immature cells. Positive identification of these immature cells has not been made. They have not been identified as either myeloblasts or megaloblasts; it seems likely

they fall into the category of primitive blastic cells. After treatment with PGA the marrow cannot be distinguished from that found in normal mice.

In a few of the animals small areas of necrosis were present in the liver. Both in this experiment and in preliminary experiments these areas of necrosis had been found in animals which had died before the development of the PGA deficiency as evidenced by leucopenia and anemia.

Discussion. There is general agreement that PGA deficiency produces a reduction of all circulating elements in the blood streams of various animals and that this reduction may be corrected by the administration of PGA. The appearance of immature elements in the peripheral blood has not been described. Reports on the histologic findings have been variable. Bethell, Swenseid, and Rosenman² using rats found that the nucleated cell count of the marrow fell slightly below normal. The majority of the marrow cells were undifferentiated primitive forms, with relative increase in myeloblasts and early erythroblasts. Myelocytes, late erythroblasts and megakaryocytes practically disappeared. The situation could be corrected by extracts of liver or yeast containing folic acid. Gross, Axelrod and Bosse³ using rats, stated that the marrow findings varied from almost complete aplasia to intense hyperplasia and concluded that the pathologic changes included progressive hypoplasia of the bone marrow. Endicott, Daft and Ott,⁴ using rats, described a decrease of all elements of the marrow, lymphoid exhaustion of the spleen, and absence of splenic hematopoiesis. They observed a pancytopenia of the marrow without maturation arrest or hyperplasia of immature elements. Doan,⁵ working with monkeys, described hypoplasia of the marrow with result-

² Bethell, F. H., Swenseid, M. E., and Rosenman, R. H., *J. Clin. Invest.*, 1944, **23**, 926.

³ Gross, P., Axelrod, A. E., and Bosse, M. D., *Am. J. Med. Sci.*, 1944, **208**, 642.

⁴ Endicott, K. M., Daft, F. S., and Ott, M., *Arch. Path.*, 1945, **40**, 364.

⁵ Doan, C. A., *Am. J. Med. Sci.*, 1946, **212**, 257.

The amount of thrombin formed during this period was measured by the addition of fibrinogen and by observing the time required for a clot to form (vertical axis, Fig. 1). This observed time is an inverse measure of the amount of thrombin formed in the first reaction. The rate of thrombin production is proportional to the concentration of the Ac-globulin in the reacting medium. Thus, it may be seen that there is a definite decrease in serum Ac-globulin activity from the time that the blood was drawn. Within 300 minutes after blood was drawn less than one-third of the Ac-globulin activity remained. On standing at room temperature the loss of serum Ac-globulin activity was considerably more pronounced (not shown in Fig. 1).

One would naturally like to offer an explanation for these observed variations, but sufficient information is not available at this time. It has been shown that relatively large

amounts of thrombin (30 units per cc or more) partially destroy the Ac-globulin activity of oxalated bovine plasma.¹ It is therefore possible that the instability of serum Ac-globulin in certain species may be due to the action of thrombin. Since the amount of thrombin present during and immediately following the clotting reaction is determined by the rate of thrombin formation and by the effective concentration of antithrombin, variations of these factors from one species to another may account for the differences in the stability of Ac-globulin activity. The stability of serum Ac-globulin may also be an inherent characteristic of the substance for each species.

The similarity in the stability of human serum Ac-globulin and that of Factor VI described by Owren⁴ tends to confirm the suggestion that these two substances are identical.

16674

Effects of Diethylcholine and Ethionine on Growth Rate, Liver Fat, and Kidney Degeneration of Rats.

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It is now well established that a dietary deficiency of choline and methionine leads to an accumulation of fat in the liver, to the development of hemorrhagic degeneration of the kidney, and to a failure of young animals to grow on diets containing homocystine but lacking in labile methyl groups. The lipotropic action and kidney protection have been ascribed to the action of intact choline or choline-like molecules or their precursors, whereas growth on a low methionine diet containing homocystine is dependent upon the

availability of labile methyl groups.

Diethylmethylhydroxyethyl ammonium chloride (diethyl choline)¹ and ethionine² have been reported to be toxic to rats on low methionine diets. These compounds are ethyl homologs of choline and methionine respectively, and their toxicity raised interesting questions as to the mechanisms by which they may interfere either with fat metabolism or with transmethylation. * The present experiments were devised to investigate in the rat the influence of these two compounds on the three criteria of inadequate choline or methyl group intake—growth on a diet containing

* The data are taken from a thesis presented by Virginia L. Hardwick to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.

¹ Moyer, A. W., and du Vigneaud, V., *J. Biol. Chem.*, 1942, 143, 373.

² Dyer, H. M., *J. Biol. Chem.*, 1938, 124, 519.

Stability of Serum Ac-Globulin.*

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Serum Ac-globulin is a clotting factor found in the globulin fraction of blood serum^{1,2} which accelerates the change of prothrombin to thrombin. This factor is present in plasma in a much less active form, plasma Ac-globulin,³⁻⁷ which serves as a precursor to serum Ac-globulin. The role of Ac-globulin in the total clotting reaction has been described as follows:^{1,2} The clotting reaction is initiated by thromboplastin, which, in the presence of calcium ions, interacts with prothrombin to form thrombin. The thrombin in turn alters the plasma Ac-globulin so that it becomes serum Ac-globulin. The latter then intensifies the reaction between thromboplastin and prothrombin. Clotting is then accomplished by the action of thrombin on fibrinogen.

In the developmental work on Ac-globulin, oxalated bovine plasma was used as a potent stable source of plasma Ac-globulin⁸ and bovine serum¹ a stable source of serum Ac-globulin. In working with the sera of other species involving man, dog, and guinea pig, we have found that while plasma Ac-globulin is relatively stable, serum Ac-globulin is inactivated and largely disappears within a

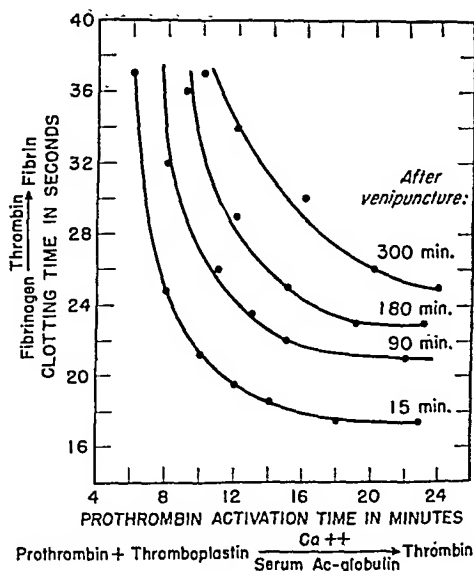


FIG. 1.
Change in Ac-globulin activity of human serum stored at 5°C.

few hours after the blood is drawn. Rabbit serum Ac-globulin is similar to bovine serum Ac-globulin in that it remains stable for longer periods of time.

In the study of the stability of serum Ac-globulin, blood was drawn and immediately centrifuged at 3,000 rpm for 10 minutes, and the serum was removed and stored at 5°C. Samples were analyzed for serum Ac-globulin by methods elsewhere described¹ after storage for periods of 15, 90, 180 and 300 minutes, as indicated in Fig. 1. In this procedure, human serum diluted 1.400 times was incubated for varying time intervals (as indicated on the horizontal axis, Fig. 1) with purified bovine prothrombin⁹ and with optimal concentrations of thromboplastin and calcium.

* Aided by a grant from the United States Public Health Service.

¹ Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1948, **152**, 567.

² Ware, A. G., Murphy, R. C., and Seegers, W. H., *Science*, 1947, **106**, 618.

³ Fantl, P., and Nance, M., *Nature*, 1946, **158**, 708.

⁴ Owren, P. A., *The coagulation of blood. Investigations on a new clotting factor*, Oslo, 1947.

⁵ Owren, P. A., *Lancet*, 1947, **1**, 446.

⁶ Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **160**, 231.

⁷ Ware, A. G., Guest, M. M., and Seegers, W. H., *Science*, 1947, **106**, 41.

⁸ Murphy, R. C., Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1947, **151**, 338.

⁹ Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, **174**, 565.

A most outstanding result apparent from Table II is the fact that the total liver choline (or its ethyl analogues) is very markedly reduced in the animals on homocystine plus diethyl choline (Group III) and on ethionine (Group VI).

Discussion. It is of special interest that ethionine should show a lipotropic action and a protection against kidney hemorrhage, since it has no methyl groups and does not have a structure which can reasonably be considered to substitute for choline in phospholipids. It does not seem likely that the lipotropic and antihemorrhagic action of ethionine can be explained on the basis of a transethylation to ethanolamine to form an ethyl analogue of choline, since such choline analogues were not found in the livers of the animals of Group VI.

The possibility that diethyl choline exerts its lipotropic action by virtue of its structural relationship to choline and consequent incorporation into phospholipid would seem to be supported by the observation of McArthur,⁶ who showed the presence of administered triethyl choline in the liver phospholipids of rats. However, in our experiments we found very much lower than normal amounts of choline or its analogues in the livers of animals fed homocystine plus diethyl choline (Group III).

It is therefore concluded that the action of ethionine and diethyl choline in preventing fatty livers and kidney hemorrhage of rats on a low choline and low methionine diet probably does not involve their incorporation in whole or in part into phospholipids.

Summary. The toxic actions of ethionine on the growth of rats and the protective action of methionine reported by Dyer² has been confirmed. It has been shown that ethionine prevents the accumulation of liver fat and the hemorrhagic kidney degeneration on low methionine, low choline diets. The diethyl analogue of choline also prevents the fatty livers and kidney lesions of rats on similar diets. The action of these compounds does not appear to involve their direct participation in phospholipid formation.

TABLE II.
Weight Changes, Liver Fat Content, Liver Choline Content and Kidney Hemorrhage in Rats.

Group	Diet	No. of animals	Avg initial wt (g)	Avg food eaten per day (g)	Wt gain per day (g)	Liver fat % dry wt*	Liver ethionine mg/g	Kidney damage
I	Homocystine	14	59	6.2	-1.0	41.0 ± 2.5	2.1	+
II	Homocystine + choline	13	61	8.1	1.5	18.8 ± 1.4	2.8	+
III	Homocystine + diethyletholine	10	57	5.0	-0.7	8.4 ± 1.0	0.1	+
IV	Homocystine + choline + diethyletholine	10	68	7.7	1.0	13.8 ± 1.1	2.9	+
V	Methionine	25	63	9.4	1.6	14.3 ± 1.1	1.8	+
VI	Ethionine	6	60	2.8	-2.6	9.8 ± 1.4	0.2	+
VII	Ethionine + methionine	5	67	7.2	1.1	23.4 ± 1.4	3.6	+
VIII	Stock	10	59	11.0	3.3	7.4 ± 0.3	2.3	-

* Including the standard error of the mean.

† Including the monoethyl, diethyl, and triethyl analogues.

⁶ McArthur, C. S., *Science*, 1946, 104, 222.

TABLE I.
Constitution of the Basic Diet.

Constituent	%
Arachin	18
Cerelose	65.2
Osborne and Mendel salts	4
Crisco and Lard*	10
Vitamin mixture†	2
Supplement	0.8

* The Crisco and lard was fortified with 6,000,000 U.S.P. units of vitamin A, 85,000 units of vitamin D, and 0.5 mg of alpha tocopherol per g of fat.

† The vitamin mixture had the following composition: cerelose, 95 g; riboflavin, 50 mg; thiamin hydrochloride, 25 mg; pyridoxine, 25 mg; nicotinic acid, 100 mg; calcium pantothenate, 50 mg; and inositol, 5 mg.

homocystine, deposition of liver fat, and hemorrhagic degeneration of the kidneys.

Methods. Diethyl choline was prepared by treating diethylaminoethanol in ether with one equivalent of methyl chloride at -50°C and allowing the condensation to proceed at room temperature at high pressure. Homocystine was prepared by the method of Patterson and du Vigneaud.³ Ethionine was prepared from S-benzylhomocysteine according to the method of Dyer.²

The basic diet used had the composition shown in Table I. Arachin was selected for the protein of the basic diet since it has been shown to contain limited amounts of methionine. It was prepared from peanut meal by the method of Johns and Jones⁴ and was found to contain 0.5% methionine. All diets were supplemented with 0.8% homocystine, ethionine, or methionine as indicated in the experimental protocols. Choline or diethylcholine were administered daily by mouth with a 0.25 ml syringe and a rounded needle. The animals objected to the administration of the supplements, but with some practice this could be achieved without loss. Rats of the University of Southern California strain weighing between 50 and 70 g were employed throughout. The experiments were continued for 12 days with the food consumption and weight changes being determined every second day.

³ Patterson, W. I., and du Vigneaud, V., *J. Biol. Chem.*, 1935, **111**, 393.

⁴ Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1916, **28**, 77.

Liver lipids were determined by extracting the dried samples with diethyl ether in continuous extractors, the total lipids being determined gravimetrically. The choline in the liver lipids were determined by the Reinecke salt method of Engle⁵ after hydrolysis with barium hydroxide. This method does not distinguish between choline and its monoethyl, diethyl or triethyl analogues. The kidneys were decapsulated and fixed in Zenkers solution, and sections embedded, sectioned and stained with iron hematoxylin.

Results. The results of the experiments are given in Table I. The rats on the unsupplemented homocystine diet (I) showed the expected weight loss, high liver fat content, and extensive kidney hemorrhage. Supplementation of this diet with choline (II) or substitution of methionine for homocystine (V) resulted in growth, lowered liver fat levels and minimal kidney pathology as anticipated from many previous studies. Growth with the methionine diet was somewhat superior to that with homocystine plus choline, but was considerably inferior to that obtained with the stock diet (VIII). This is no doubt to be attributed to deficiencies in amino acids other than methionine in arachin. Supplementation of the homocystine diet with diethyl choline did not permit growth, but a strong lipotropic action and a prevention of kidney hemorrhage were apparent. This is in accordance with previous observations.¹ However, the toxicity on growing rats reported for this compound¹ was not apparent in the present studies. The animals receiving both choline and diethyl choline (Group IV) did not differ significantly from those receiving choline alone.

The animals receiving ethionine (VI) lost weight more rapidly than did those receiving homocystine. This toxic effect of ethionine was partially prevented by addition of methionine. The toxic effect of ethionine on rats and the protection by methionine has already been reported by Dyer.² It is of utmost interest, however, that the accumulation of liver fat and the development of kidney hemorrhage did not develop on the ethionine diet.

⁵ Engle, R. W., *J. Biol. Chem.*, 1942, **144**, 701.

TABLE I.
Toxicity of Various Sodium Chloride Solutions Administered Intravenously at Different Rates.

% NaCl	Rate of administration cc/minute 4.0-3.5		2.9-2.5		2.4-2.0		1.9-1.5		1.4-1.0		0.9-0.5		0.5-0.2	
	cc/20 g D*		cc/20 g D*		cc/20 g D*		cc/20 g D*		cc/20 g D*		cc/20 g D*		cc/20 g D*	
0	4.7†	4/4	4.6†	3/3	5.1	1/1	4.5†	1/1	4.2†	1/1	3.9†	1/1	4.6†	2/2
0.425	5	3/10	5	0/9	5	0/6	5	0/3	5	0/2	5	0/1		
0.85	5	4/6	5	2/8	5	0/3	5	0/4	"Safe" range		5.0	0/6	5.0	0/1
1.70	3.9†	1/1	4.0†	2/2					4.2†	1/1	5.0	1/3	5.0	1/3
2.55	2.2†	1/1					1.8†	1/1	2.6†	1/1	2.9†	3/3	5.1	2/2

* D = $\frac{\text{No. dead}}{\text{No. dosed}}$

† Death before 5 cc injected.

‡ Two of these animals received 7 cc per 20 g.

16676

Failure of Xanthopterin to Influence Hematopoiesis and Growth in Rats.*

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Tschesche and Wolf¹ reported that rats fed goats' milk develop an anemia which responds to xanthopterin. Simmons and Norris² and Simmons³ observed that anemia in Chinook salmon responds to injections of xanthopterin. Totter and Day⁴ reported that in rats with the deficiency induced by feeding a purified diet containing succinylsulfathiazole, administration of 20 µg of xanthopterin per day caused a leukocytosis and a marked weight

gain, but in subsequent experiments they failed to confirm this observation. Wright and Welch,⁵ Daft and Sebrell,⁶ and Axelrod, *et al.*,⁷ also failed to note any effect when xanthopterin was fed to rats deficient in pteroylglutamic acid (PGA). Totter and co-workers^{8,9} reported that favorable hematopoietic responses occur when xanthopterin is given to vitamin M-deficient monkeys. In this laboratory, 2 macrocytically anemic swine have shown a submaximal hematopoietic response to large parenteral doses of xanthopterin (10 mg daily),¹⁰ a phenomenon which is being investigated further.

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¹ Tschesche, R., and Wolf, H. G., *Ztsch. f. physiol. Chem.*, 1937, **248**, 34.

² Simmons, R. W., and Norris, E. R., *J. Biol. Chem.*, 1941, **140**, 679.

³ Norris, E. R., and Simmons, R. W., *J. Biol. Chem.*, 1945, **158**, 449.

⁴ Totter, J. R., and Day, P. L., *J. Biol. Chem.*, 1943, **147**, 257.

⁵ Wright, L. D., and Welch, A. D., *Science*, 1943, **98**, 179.

⁶ Daft, F. S., and Sebrell, W. H., *Public Health Rep.*, 1943, **58**, 1542.

⁷ Axelrod, A. E., Gross, P., Bosse, M. D., and Livingly, K. L., *J. Biol. Chem.*, 1943, **148**, 721.

⁸ Totter, J. R., Shukers, C. F., Kolson, J., Mims, V., and Day, P. L., *Fed. Proc.*, 1943, **2**, 72.

⁹ Totter, J. R., Shukers, C. F., Kolson, J., Mims, V., and Day, P. L., *J. Biol. Chem.*, 1944, **152**, 147.

Intravenous Saline Tolerance in Mice.

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In the course of investigating the comparative toxicity of certain sparingly soluble compounds in mice, it was necessary to inject as much as 5 cc per 20 g of body weight of hypotonic and isotonic solutions. In order to determine the effects of injecting such large volumes without the influence of any drug, the intravenous toxicity of sodium chloride solutions at different osmotic concentrations was investigated. In addition the rate of injection was varied to determine the maximum speed permissible for safe administration.

Male mice of the CF 1 strain, weighing from 19 to 30 g were used and all animals surviving the initial injection were observed one week for delayed deaths. The solutions were administered into one of the lateral veins of the tail at a given rate by means of a constant speed infusion pump.

In the first experiment physiological saline (0.85% NaCl, 0.15N) was administered until death occurred. At the rate of 0.9 cc per minute, an average of $6.9 \pm 0.71^{\dagger}$ cc per 20 g was found to be the fatal dose. Although increasing the rate of administration above 1 cc per minute (1 to 2.5 cc per min.) appeared to decrease the fatal dose to $5.9 \pm 0.63^{\dagger}$ per 20 g, the difference between the 2 sets of data is not significant. After establishing the fatal dose for physiological saline, a second experiment was carried out in which various concentrations of sodium chloride were injected at different rates until the animals received 5 cc per 20 g or less if death occurred. Accordingly the animals were divided into 5 groups receiving one of the following solutions: distilled water, 0.425%,

0.85%, 1.70%, or 2.55% sodium chloride at rates ranging from 0.2 to 4.0 cc per minute. As is shown in Table I, half physiological saline (0.425%, 0.073N) is the least toxic; 5 cc per 20 g was tolerated by all animals at a maximum rate of 3.50 cc per minute. Increasing the rate above this point results in death. As the salt concentration was increased, tolerance to 5 cc per 20 g was reduced. With physiological saline, this volume was tolerated at a maximum rate of 2.5 cc per minute, but in the case of distilled water, 1.70 or 2.55% sodium chloride, 5 cc per 20 g could not be administered safely, for some deaths occurred even at the slowest rates. It would hardly be practical to administer 5 cc per 20 g at rates below 0.2 cc per minute because of the time required for the injection.

Both physiological and half physiological saline are well tolerated under the circumstances of this experiment whereas the hypertonic solutions are not "safe" since deaths occurred even at the slowest rates of administration. Although the half physiological saline appears to be slightly less toxic than the physiological saline this difference is not statistically significant. Therefore, when large volumes (5 cc per 20 g body weight) are required in intravenous toxicity studies in mice, it would seem most logical to use solutions with an osmotic concentration between the range of 0.073N to 0.15N. This is equivalent to 0.425% to 0.85% NaCl. In our studies we adjusted the solutions of the sparingly soluble compounds to 0.073N and injected up to 5 cc per 20 g at a rate of 1.5 cc per minute.

Summary. Mice tolerated intravenous injections of 5 cc per 20 g body weight of half physiological and physiological saline at maximum rates of 3.5 and 2.5 cc per minute respectively. The average lethal dose for physiological saline given at a rate of 0.9 cc per minute is 6.9 cc per 20 g.

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‡ Standard error.

pairment of growth, leukocyte and hemoglobin levels were determined. Twelve severely leukopenic rats were assigned to group II. In these rats the effects of xanthopterin and of PGA on leukopoiesis and growth were studied. Each of 6 rats was injected with 50 μ g of PGA daily for 5 days. Into the remaining 6 rats, xanthopterin was injected daily for 5 days; the daily dosage was 100 μ g in one rat, 200 μ g in 4 rats, and 400 μ g in the sixth rat. Leukocyte counts and weighings were made at 3-day intervals. The positive effects of PGA and the lack of effect of xanthopterin are considered under *Results*.

The remaining 21 rats, of the group given the purified diet plus 2% succinylsulfathiazole, composed group III. In this group a study was made of the effects of xanthopterin and of PGA on hemoglobin synthesis, as well as on growth and leukopoiesis. Under these conditions, severe anemia failed to develop during a period of 28 days. However, an anemia was induced by means of hemorrhage, using a technic similar to that described by Kornberg *et al.*¹⁶ Blood, in an amount equivalent to approximately 2% of the weight of the rat, was removed at each bleeding. Sufficiently low hemoglobin levels were produced by 2 to 4 bleedings of each rat, carried out at intervals of 2 days. Two rats died during the course of the bleeding, and 2 rats died 2 days following the last bleeding. Seventeen rats were available for experiment, of which 4 served as untreated controls. Each of 4 rats was given parenterally 50 μ g of PGA daily for 5 days. Nine rats were given xanthopterin parenterally; 5 of the 9 were given 100 μ g each, daily for 5 days, while 4 were given twice this dose. Weighings, leukocyte counts and hemoglobin measurements were done at 3-day intervals. Hemoglobin was determined as oxyhemoglobin using a Klett-Sumerson photoelectric colorimeter. The findings are presented in Figure 1 and are discussed under *Results*.

Results. Group I (1% sulfathiazole). In rats given a purified diet containing succinyl sulfathiazole, anemia is rarely seen. How-

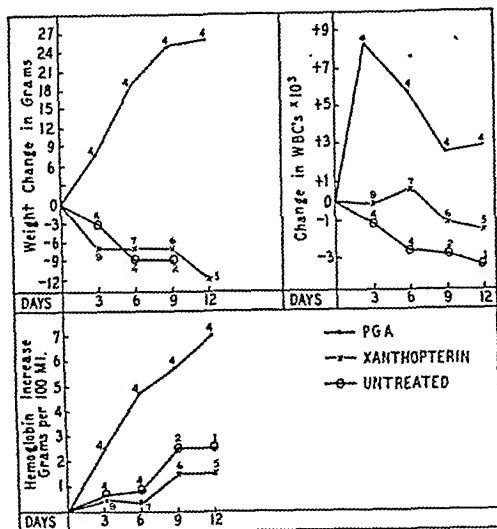


FIG. 1.

Changes in hemoglobin levels, leukocyte counts, and weight in rats fed a purified diet containing 2% succinylsulfathiazole and bled 2 to 4 times. One group received pteroylglutamic acid, one group received xanthopterin, and a third group served as the untreated control. The numbers at each point in the figure represent the number of surviving rats represented in the average.

ever, the use of sulfathiazole in a purified diet induced, during a 7-week period, an anemia in 8 of 10 surviving rats.

The data presented here indicate clearly that a markedly variable *hemolytic anemia* has occurred in rats fed sulfathiazole in a purified diet. Reticulocytoses of 10 to 25% were noted in many of the anemic rats before therapy. Those rats in which anemia developed earlier did not show leukopenia. Therefore, leukopoiesis and erythropoiesis, as judged by reticulocytosis, certainly was occurring. It is evident that the anemia was due to factors other than one related directly to the lack of an essential hematopoietic factor or factors. The feces of many of the rats were observed to contain great amounts of bile pigments. One rat (No. 69), while receiving a total dose of 300 μ g of PGA parenterally, showed a decrease in the number of circulating erythrocytes from 4.0 million to 1.7 million per cmm in 6 days, yet during this same period, the leukocyte count increased from 3,700 to 68,500 per cmm; on the sixth day, a reticulocytosis in excess of 80% was present. The marked anemia, with

¹⁶ Kornberg, A., Tabor, H., and Sebrell, W. H., *Am. J. Physiol.*, 1944, 142, 604.

Cerecedo and his associates^{11,12} found that xanthopterin, like PGA, when added to highly purified diets fed to mice or rats, causes a significant improvement in lactation performance. Fraenkel and Blewett¹³ have reported that the growth of the larvae of the insect, *Ephestia kuehniella*, on an artificial diet which contains all the known factors of the vitamin B-complex in pure form, is markedly accelerated by the addition of PGA, and that xanthopterin, in concentrations about 1000-times greater, has a comparable effect.

Recently Norris and Majnarich^{14,15} have studied the effects of xanthopterin in experiments conducted both *in vitro* and *in vivo*. Studies *in vitro* involved the use of cultures of bone marrow from rats and other animals. Xanthopterin in low concentrations was reported to cause an increase in the rate of red and white cell proliferation. In rats fed a purified diet containing sulfathiazole (1%), it was claimed that xanthopterin is hematopoietically active. They reported that xanthopterin *in vitro* and *in vivo*, is more effective than PGA, since it appeared to act in smaller doses and more rapidly. They concluded that bone marrow cannot use PGA directly in hematopoiesis while xanthopterin can be so utilized.

Findings with xanthopterin have been so variable, and the claims made by Norris and Majnarich are so remarkable, that in this study a further attempt has been made to determine whether xanthopterin can play a role in hematopoiesis and growth in rats. The data obtained do not support the conclusions drawn by Norris and Majnarich.

Methods. Weanling female albino rats† were kept singly in cages with wire mesh

floors. The basal diet had the following composition (in g per 100 g): purified casein, 20; glucose ("Cerelease"), 48.7; hydrogenated vegetable oil ("Primex"), 18; corn oil, containing vitamins A and D,§ 2; salt mixture (U.S.P.X. No. 2), 2.8; accessory salts|| 0.2; cellulose ("Cellu-flour"), 5; choline chloride, 0.2; inositol, 0.1; thiamine hydrochloride and pyridoxine hydrochloride, each, 0.0005; riboflavin, 0.001; nicotinamide, 0.005; calcium pantothenate, 0.0055; ascorbic acid, 0.01; 2-methyl-1,4-naphthoquinone, 0.001; biotin, 0.00001.

Group I. Sixteen of the weanling rats were placed on the above basal diet with 1% sulfathiazole incorporated. At the end of 28 days, 3 rats were dead and 3 rats showed anemia without leukopenia. During the following 21 days, 5 more rats became anemic. In those rats that developed anemia during the latter period, leukopenia also was present. During this 21-day period, 3 rats died before developing an anemia of a level suitable for experiment; at the end of this period an erythrocyte level exceeding 5,000,000 per cm was found in 2 rats. Each of 3 rats (No. 78, No. 80 and No. 82) was given 100 µg of xanthopterin parenterally per day for 6 days; also one (No. 81) was given 200 µg per day, and another (No. 76) was given 400 µg per day, for 6 daily injections. Each of 5 rats (No. 69, No. 73, No. 80, No. 82, and No. 84) was given 50 µg of PGA daily for 6 injections; 2 of these rats (No. 80 and No. 82) previously had been treated with xanthopterin. The variable effects of xanthopterin and PGA are considered in the section entitled *Results*.

Groups II and III. Forty rats were placed on the basal diet in which 2% succinylsulfathiazole was incorporated. At the end of 28 days, when the rats showed marked im-

¹⁰ Pritchard, J. A., Welch, A. D., and Heinle, R. W., unpublished data.

¹¹ Mirone, L., and Cerecedo, L. R., *Arch. Biochem.*, 1948, 15, 324.

¹² Sica, A. J., Allgeier, A. M., and Cerecedo, L. R., *Arch. Biochem.*, 1948, 18, 119.

¹³ Fraenkel, G., and Blewett, M., *Biochem. J.*, 1947, 41, xviii.

¹⁴ Norris, E. A., and Majnarich, J. J., *Am. J. Physiol.*, 1948, 152, 175.

¹⁵ Norris, E. A., and Majnarich, J. J., *Am. J. Physiol.*, 1948, 152, 178.

† Obtained from Blaine Biological Gardens, Media, Pa.

§ Two grams of the corn oil preparation contained vitamin A, about 4500 units and vitamin D, about 560 units.

|| Accessory salts contained in 0.2 g (expressed in mg): KCl, 100; NaCl, 87.2; FeSO₄, 10; MnSO₄, 1; ZnSO₄ · H₂O, 1; NaI, 0.3; NaF, 0.1; CoCl₂ · 6H₂O, 0.01.

per 100 ml at the start of the observation period. The average hemoglobin level closely paralleled that of the xanthopterin-treated anemic rats during the 12-day observation period. Only 1 rat was alive at the end of 12 days. Weight and leukocyte changes also were similar to those noted in the xanthopterin-treated rats.

Discussion. Norris and Majnarich¹⁵ have not indicated the mechanism of production of the anemia they observed in rats fed a purified diet containing sulfathiazole. Sulfathiazole has been reported by Richardson¹⁷ to cause hemolytic anemia and the hemolytic anemia-producing properties of related sulfonamides have been studied by Latven and Welch.¹⁸ That such a hemolytic process operated in the rats of Norris and Majnarich¹⁵ is indicated by the production of an anemia within 22 days after the diet was started, and by the rapid fall in erythrocyte counts noted after the brief rise following therapy. Against a hemolytic process are the apparently very low reticulocyte levels which were reported by them; it would seem certain, however, that a measurable erythrocytic response following therapy in any type of severe anemia would produce greater reticulocyte responses than the 0.8 and 1% maximum values that they observed. It is suggested that the reticulo-

cyte counts reported by Norris and Majnarich¹⁵ are in error.

The effects shown here of PGA and xanthopterin in rats made anemic by feeding a purified diet containing sulfathiazole do not confirm the observations of Norris and Majnarich.¹⁵ No positive leukocytic responses were noted after therapy with xanthopterin, although marked responses occurred after the administration of PGA. Doses of xanthopterin larger than the doses they used failed to produce the distinct increases in circulating erythrocytes that they recorded. Highly variable results also were obtained when PGA was given to those rats fed a purified diet containing sulfathiazole; however, those PGA-deficient rats made anemic by hemorrhage, and then given PGA, all showed nearly equal, marked erythropoietic responses.

Summary. 1. A purified diet containing sulfathiazole produced a hemolytic anemia of variable intensity which could not be completely reversed either by pteroylglutamic acid or by xanthopterin in any of the doses used.

2. Rats fed a purified diet containing succinylsulfathiazole developed leukopenia and an inhibition of growth which responded to PGA, but not to xanthopterin.

3. In similar rats fed a purified diet containing succinylsulfathiazole, but made anemic by repeated hemorrhage, PGA induced definite erythropoietic, leukopoietic and growth responses, while xanthopterin was inactive.

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Stripping Film Technics for Histological Autoradiographs.

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There are now three general technics of autoradiography. The first method developed was that of placing the specimen in contact with a photographic plate, removing the specimen, and developing the plate. London¹

studied anatomical specimens in 1904 and Lacassagne and Lattes,² histological specimens

¹ London, E. S., *Arch. D'elec. Med.*, 1904, **12**, 363.

² Lacassagne, M. A., and Lattes, J. S., *Compt. rend. Acad. Sci.*, 1924, **178**, 488.

a marked leukocytosis and reticulocytosis, was maintained. The rat died on the twelfth day after the start of therapy. A similar fall in erythrocyte count was noted in a rat (No. 76) which received a total dose of 2400 μ g of xanthopterin. The red blood cell level fell from 4.0 million to 2.9 million per cmm; a reticulocytosis of 20% was present on the sixth day. During this 6-day period, the leukocyte count increased from 4,900 to only 6,700 per cmm. The rat died 15 days after treatment with xanthopterin was started.

Four rats (No. 78, 80, 81 and 82) showed a slight increase in the number of circulating erythrocytes after treatment with xanthopterin. During the 9 days following the start of xanthopterin therapy, an average increase in erythrocytes of 1.4 million per cmm occurred; the largest increase was 1.8 million (No. 80). The leukocyte counts fell in all 4 rats. No rat receiving xanthopterin showed a positive growth response.

In the 5 rats given PGA, variable effects on growth and circulating erythrocyte levels were obtained. A marked leukocytosis occurred in all PGA-treated rats. The largest increase in erythrocyte count was 3.3 million per cmm (No. 82). The small increase in erythrocyte counts that occurred in rats given xanthopterin, did not occur earlier than in those given PGA.

Group II (2% succinylsulfathiazole). The effect of xanthopterin and of PGA on growth and leukopoiesis were compared in leukopenic rats with arrested growth. Six rats given a daily dose of 50 μ g of PGA parenterally for 5 days showed favorable leukocyte and growth responses during the 12 days of study. Six days after the start of therapy an average increase in white blood cells of 12,900 per cmm was noted. Six rats receiving 2 to 8 times as much xanthopterin parenterally showed an average increase of 1,300 per cmm. At the end of 12 days those rats given PGA all were alive and an average gain in weight of 21 g was observed. The xanthopterin-treated rats showed an average loss in weight of 11 g during this same period. One rat given xanthopterin died on the sixth day. No differences were observed in leukocyte and

growth responses to different dose levels of xanthopterin. "Spectacled" eyes and ruffled fur developed in rats during the period of administration of xanthopterin. The lack of effect of xanthopterin on growth and leukopoiesis is similar to that previously observed.⁵⁻⁷

Group III (2% succinylsulfathiazole plus hemorrhage). It was felt that the study of the comparative effects of xanthopterin and PGA on erythropoiesis could be conducted more satisfactorily in deficient rats made anemic by hemorrhage than in rats with an anemia due to an active hemolytic process.

Definite hematopoietic and growth responses followed therapy with PGA. Little difference between the responses occurring in the xanthopterin-treated anemic group and in the untreated anemic control group was noted.

In the 4 rats given PGA, the average hemoglobin concentration at the start of therapy was 6.6 g per 100 ml of blood. During the 12 days after the start of therapy, an increase of 7.1 g to an average concentration of 13.7 g per 100 ml occurred. This was accompanied by an average growth response of 26 g. An average increase in leukocytes of 8,300 per cmm was noted on the third day. All the PGA-treated rats were alive at the end of the experiment.

Nine anemic rats were given xanthopterin in doses 2 to 4 times larger than the dose of PGA used. The average hemoglobin concentration at the start of therapy was 6.3 g per 100 ml of blood. The hemoglobin concentration at the end of 12 days in the 5 surviving rats was 8.1 g. No favorable leukopoietic response was noted. Loss of weight continued in spite of xanthopterin therapy. The 5 surviving rats lost an average of 12 g during the 12 days following the start of therapy. "Spectacled" eyes and ruffled fur developed in spite of the administration of the pterin. Three of the 5 surviving rats died during the 3 days following the last blood determinations. No significant difference was noted between those rats given 200 μ g and those rats given 100 μ g of xanthopterin per day.

Four anemic rats were not treated. Their average hemoglobin concentration was 8.2 g

The base, *i.e.*, the 10 μ cellulose ester film, is required to hold the low rigidity emulsion. This is especially true of the heavily loaded NTB emulsion with its low gelatin content. The rigidity of the emulsion has been further reduced in comparison to the ordinary emulsion by having been reduced in thickness to increase resolution of the betagraphs. Most of the satisfactory emulsions which we have used have been approximately ten micra although other thicknesses can be obtained.

The background grain problem. While the NTB emulsion on plates has an extremely low fog background, the same emulsion when stripped from the support may contain considerable fog, depending upon the stripping conditions. This appears as long streaks of single grain width, streaks of multiple grain width, and splotches. The streaks of single grain and multiple grain width are always on the surface and can be differentiated from nuclear tracks which dive at various angles into the emulsion. Some nuclear tracks do occur occasionally on the surface, but their length and grain density, as determined by those that do dive into the emulsion, differentiate them from the surface streaks.

However, the splotches cannot be differentiated from a random distribution of grains produced by beta particles. Therefore, if one wished to use stripping film for betagraphs, every precaution must be taken to prevent fog development. While we do not yet know how to eliminate all background grains, the following hints will aid in reducing them.

The usual precautions must be taken against light leakage and the presence of fogging chemicals in both the darkroom and dark storage boxes. For example, it is imperative that dark boxes, even though made of 1" wood, be painted inside and out with black paint. Unpainted wood will transmit sufficient light to fog these emulsions. Obviously, one should not touch the emulsion with the hands. Abrasion fogging can be prevented by lifting the film out of the box instead of pulling it out edgewise and by preventing contact of any non-yielding object with the emulsion. It should be emphasized that the technics of ordinary amateur or scientific photography

will not suffice for precise beta autoradiography. A few fog grains which would not greatly influence interpretation of a spectrographic plate or a photomicrograph of a histological section can lead to error in the interpretation of a betagraph under high magnification. In the latter there must either be no background fog or a known and constant quantity if one wishes even a semiquantitative estimate of the beta emitter in a histological section.

Stripping. The stripping of the base from the support is one of the greatest sources of fog. In the breaking of the bond between the base and the support, static charges are produced. If the film is stripped rapidly, these charges produce a light visible to the dark-adapted eye. This, though small in amount, will fog the emulsion as can be determined by examination under the microscope with a 1.8 mm objective. If the film is stripped slowly so that no light is observed, the fog is reduced but not eliminated. Even with this precaution, however, the static charges are retained by the film. Apparently the charges either produce latent images directly or indirectly by unperceived photons. The charges can be reduced by stripping in a humid atmosphere.

The technic used in our laboratory is as follows: A section of the film of the required size is protected in some suitable manner with a light opaque shield in addition to the usual darkroom precautions. It can be wrapped in black paper if care is taken to prevent the stiff paper from scratching the emulsion. Another method is to use a dark box with a slot in one side, the slot being covered with velvet. In either case about 1 cm is exposed. With the exposed end on the table top, emulsion side down, cut deeply but not completely through the support. (The position of the emulsion to be used for the autograph will be some distance removed from this point and protected from radiation by the shield.) Break only the support at the cut, *i.e.*, do not break the support and the base. On the break, photons are generated but the shield protects the emulsion. After the break the shield is removed and the base is stripped slowly while blowing the humid

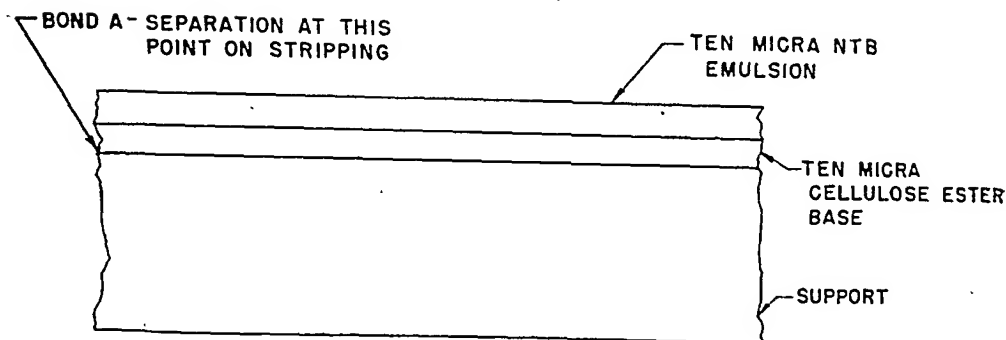


FIG. 1.

Schematic illustration of Eastman NTB stripping film used for autoradiographs.

in 1924 in this manner. In recent years Bélanger and Leblond³ developed the procedure of painting the tissue section with a photographic emulsion. Endicott⁴ and Evans⁵ independently worked out a third technic of permanently laying a tissue section on the emulsion of a plate or film. Each of these methods has its advantages and disadvantages, some of which have been discussed elsewhere.⁶

The use of stripping film has certain advantages. With the tissue mounted on the emulsion of the film stretched over a frame, back scattering is eliminated, and thus, in theory, resolution is increased. We have not as yet proved that this is of practical value. Another advantage is the ability to make autoradiographs of blood and bone marrow smears on microscope slides by the simple procedure of laying the film on the smear. With the histological specimen mounted on the photographically insensitive and water impervious base, it is separated by 10 μ from the autograph. This enables one to prevent harm to the tissue while developing and fixing and to eliminate staining of the emulsion. This separation of tissue and emulsion also enables one to identify histological bodies coincident with an alpha sunburst. Also, the separation opens the way to a semi-quantita-

tive estimation of beta emitters by counting the grains which can be seen beneath the darkly stained nuclei because of the difference in the focal plane. And since the tissue is not in contact with the emulsion chemical fogging is eliminated, an application which will probably prove to be most valuable.

Beginning in the early fall of 1947, we have developed methods of using stripping film to achieve these advantages. During this work there appeared the paper of Pelc⁷ on the use of stripping film.*

Description of the film. The stripping film was made in experimental quantities for this work by the research laboratory of the Eastman Kodak Company. Nuclear Track Emulsion B (NTB) was used as this had been found to be the most sensitive to beta particles for its grain size. It can also be used for alphagraphs. The sensitivity and fogging qualities of the emulsion on plates will be discussed in another paper. The stripping film comprises a thick cellulose ester support to one side of which has been bonded a thin film (approximately 10 μ) of another cellulose ester which serves as the base for the emulsion. A diagrammatic sketch is shown in Fig. 1. The thicker cellulose ester serves merely as a rigid support for the manufacture and transport of the base-emulsion combination, the latter being the film which is stripped.

³ Bélanger, L. F., and Leblond, C. P., *Endocrinology*, 1946, 39, 8.

⁴ Endicott, K. M., and Yagoda, H., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 170.

⁵ Evans, T. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 313.

⁶ Evans, T. C., *Nucleonics*, 1948, 2, 52.

* While this paper was in the process of declassification there appeared another paper on stripping film: "Radioautograph Technic with C14," A. M. MacDonald, Jock Cobb, and A. K. Solomon, *Science*, 1948, 107, 550.

⁷ Pelc, S. R., *Nature*, 1947, 160, 749.

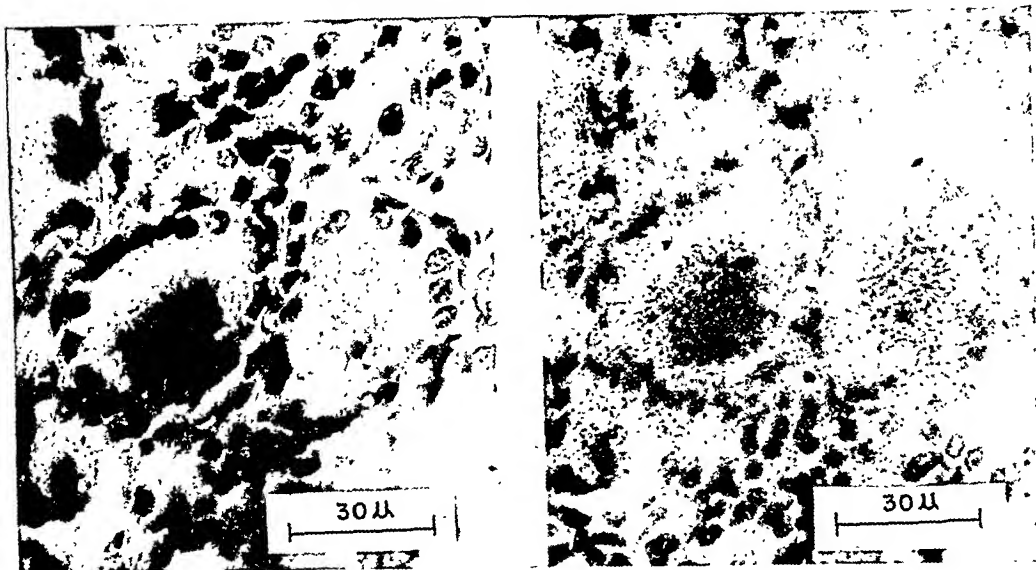


FIG. 3.

a. (left) Tissue level. b. (right) Grain level.

Photomicrographs of a thyroid tissue-autograph. A $7\ \mu$ section containing I131 from a rat sacrificed 24 hours after injection of $500\ \mu\text{c}$, exposed 17 hours on NTB stripping film.

fails to bond, the glass can be painted with egg albumin or Kodak Stripping Film Cement. The excess film can now be cut away from the tissue and autograph area if desired. The entire unstained tissue and film can be mounted in Clarite, Permunt, etc., and examined with the dark phase microscope.

However, since the developing and fixing solution slightly changes the refractive index differential of the histological bodies, *i.e.*, clears the tissue optically, the phase examination does not always give the best results. In this case the tissue and emulsion after having been placed on the slide can be stained in the usual manner. Some stain is taken up by the gelatin of the emulsion, but the gelatin content in ten micra of heavily loaded emulsion is low and the consequent uptake of hematoxylin and eosin is low in comparison with $7\ \mu$ of tissue. The background stain is insignificant and color contrast good at high magnification.

Fig. 3 shows a thyroid autograph taken with stripping film. The tissue was placed on the emulsion and later stained with hematoxylin and eosin. Fig. 3-a shows the tissue focal plane, originally photographed with a 1.8 mm objective and a 10x ocular. The

grain focal plane is shown in photograph 3-b. These illustrate a difficulty which, while it is not impossible to overcome, requires careful handling of the film. It will be seen that the entire field of each is not in good focus. This is due to uneven laying of the film on the glass slide.

Preparation of the autograph with the tissue on the cellulose ester base. If it is desired to eliminate all staining of the emulsion, the tissue can be placed on the cellulose ester base. In this procedure the base is painted with egg albumin to seal the tissue and prevent it from coming off in the developing, fixing, or staining solutions. However, as one or 2 days, is required for complete drying of the albumin, we have found it expedient in exposures under 2 days to develop and fix by placing the solutions in the $\frac{1}{8}$ " well of the Lucite frame, removing them by tipping to one side and draining. This prevents the washing off of the tissue. The preparation is dried well before placing in the staining solutions.

Since in this method the paraffin is not removed before development, the tissue is protected and clearing is prevented. After development the tissue-emulsion area is cut out

breath onto the point of bondage to discharge the static electricity. As the base-emulsion combination is stripped it should make as small an angle with the support as possible to avoid mechanical fogging due to creasing of the emulsion.

Preparation of autograph with the tissue on the emulsion. Autographs can of course be made by placing the tissue either on the emulsion or on the cellulose ester base. In the latter case, some resolution is lost because of the spatial separation of the tissue from the emulsion. For the highest resolution, the tissue is placed on the emulsion, thus decreasing to a minimum the distance between the origin of the ionizing particles and the emulsion; and at the same time the stripping film on a frame eliminates backscattering.

A 2" x 6" section of the stripped film is floated on water, the emulsion side down. It is then transferred to a Lucite frame 2" x 6" x $\frac{1}{8}$ " by placing the frame in the water beneath the film. In the center of the frame is a rectangular opening 1" x 3" which is covered with the film, exposing the emulsion inside the $\frac{1}{8}$ " well and the cellulose base on the other side. The tissue is placed in this area on either side.

The tissue section has been prepared before entering the darkroom as follows: One or more sections from a ribbon of paraffin-embedded tissue is floated on water in a petri dish on a hot plate.⁵ The water is warmed until the wrinkles have disappeared. This is now picked up by inserting a section lift under the edge of the paraffin and lifting it from the water while the remaining portion of the section hangs from the edge. The angle at which the section lift is pulled out of the water as illustrated in Fig. 2 is necessary to enable the tissue to hang freely. This manipulation is facilitated by drilling a large number of holes in the section lift to permit the water to drain from under the section. If the water drains over the edge of the lift, the current will carry the tissue section back into the petri dish.

The free end of the tissue section is placed on the wet emulsion immediately after the film is placed on the Lucite frame. With a



Fig. 2.

Removing a tissue section from water with a section lift, the perforated spoon held vertically to prevent the tissue from curling around the edge. The bottom of the tissue is touched to the photographic emulsion with the lift in the same vertical position after which the spoon is backed away horizontally from the tissue, permitting it to lie on the surface.

horizontal motion the tissue is freed from the section lift and laid flat on the film. Any excess water is permitted to drain off or is picked up by a small hand syringe. The preparation is placed in a lead lined dark box in the refrigerator for exposure.

As the film dries it becomes taut and smooth over the 1" and 3" well and the gelatin of the emulsion bonds the film to the Lucite frame. Occasionally after long exposure the film becomes loose at the end of the frame and curls. Paper clamps can be used to prevent this, but they should not be put in place until after the film has dried. If placed on earlier, the contraction of the gelatin in drying may break the film.

The paraffin is removed in the usual manner before development⁶ which is accomplished by passing the frame, film, and tissue as a single unit through the solutions. After development in D-19 at 20°C for 2 minutes, fixing in 30% hypo and washing for about 10 minutes each, the 1" x 3" film is cut out and placed on a microscope slide, the emulsion side up. When thoroughly dry the cellulose ester base usually adheres to the glass. If it

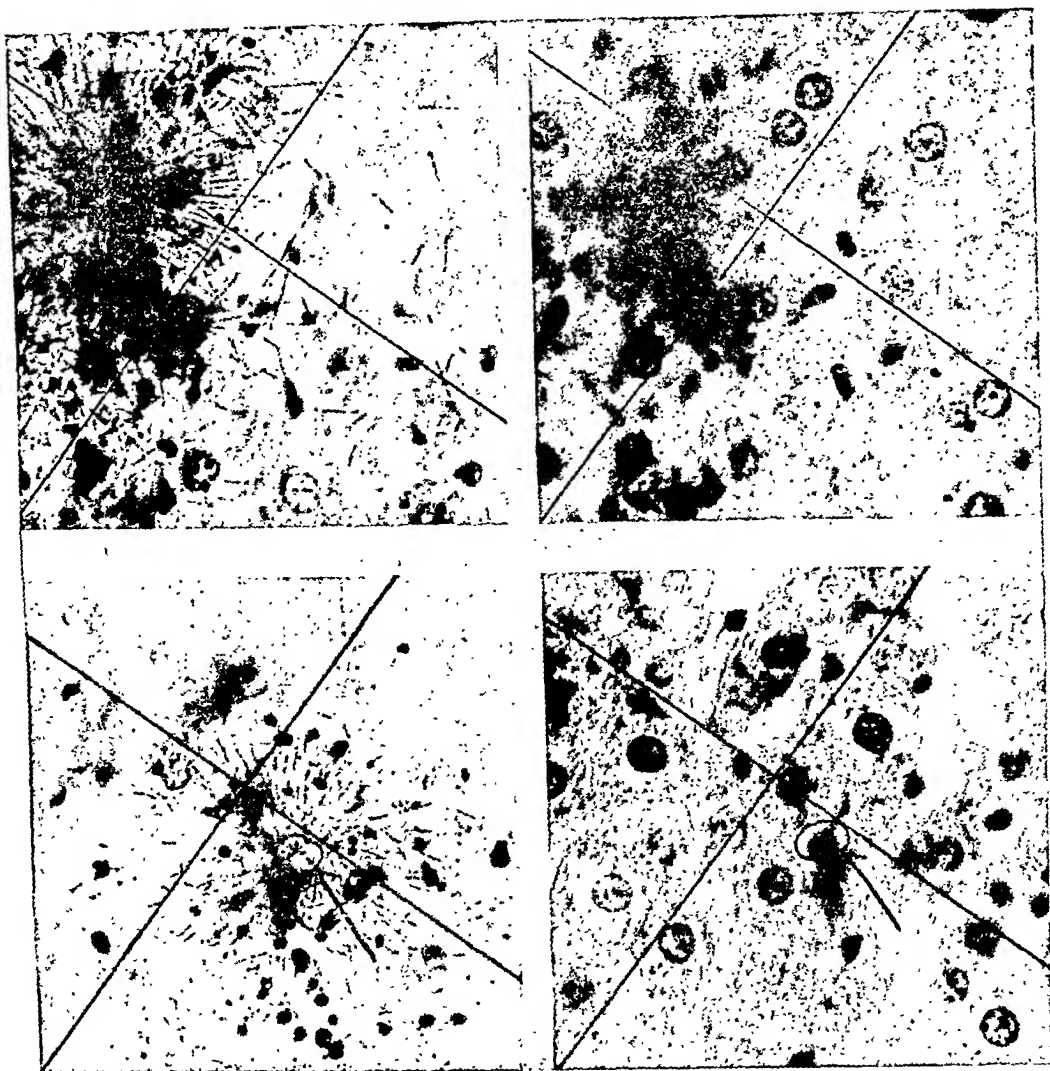


FIG. 5.

a (top left); b (top right); c (lower left); d (lower right).

Illustration of stripping film technique in identifying a histological body of rat liver containing an alpha emitter. In a and b the tissue section is on the emulsion of an NTA plate: a, track level; b, tissue level. In c and d the tissue is on the cellulose ester base of NTB stripping film; c, end of tracks in emulsion showing center of burst under a von Kupffer cell indicated in d.

an alpha emitter directly on an NTA (Nuclear Track A) plate, the former being a photograph of tracks in the emulsion, *i.e.*, just below the tissue-emulsion interface, and the latter is of the tissue directly above the tracks. Because of the high concentration of silver grains in the center of the sunburst there is insufficient illumination from below

to permit observation or photography of the histological body directly above. Since this body presumably contains the collection of the radioactive element it is of interest to identify it.

When another section from the same liver specimen was placed on the cellulose ester support of a stripped film, photographs 5-c

as described above and placed on a microscope slide, emulsion side down. The tissue is now deparaffinated in xylol and taken through the alcohol-water solutions to water and then the edges of the film sealed by dipping in molten paraffin. The tissue is then stained. The paraffin around the edges of the film is removed and the tissue-film combination mounted as described previously.

The emulsion which is protected by the aqueous-impervious paraffin and the cellulose ester is not stained, thus giving a high color differential between the tissue and the gelatin. The phase microscope can also be used without staining. The tissue intensity differentials as seen by the phase microscope have not been reduced by partial clearing, since the tissue has been protected in the developing and fixing solutions by the paraffin.

Stripping film for blood smears. The ideal blood smear for an autoradiograph has the cells separated by several micra so that the autograph of the beta emitters of one cell will not be confused with those of adjoining cells. It is difficult to make such a smear on an emulsion since the gelatin imbibes the water so quickly that the cells will not flow easily.

A thin smear is made on a microscope slide in the usual manner, stained for bright field illumination or left unstained for phase examination. In either case it is fixed for about 3 minutes with absolute methyl alcohol. After drying thoroughly, it is flooded with water and a piece of the film, emulsion side up, is floated over the smear. The

water is then drawn out from under the film with filter paper, permitting the film to settle uniformly and without wrinkles over the smear. Since the fixing solution destroys the stain properties of the leukocytes, the procedure of paraffin dipping the edges is used to protect the smear during the developing and fixing steps. After washing and drying the paraffin is cut away with a razor blade and the smear, covered with the film, is mounted under a glass slip.

Fig. 4 shows a concentration of a source of alpha particles coincident with one erythrocyte as evidenced by the center of an alpha track sunburst. This is a dark contrast phase photomicrograph of an unstained smear.

It should not be inferred from the photomicrograph that the alpha emitter is attached to the cell. It cannot yet be definitely stated whether the element concentrate is associated with the red cell in the blood stream or whether it has settled upon the cell in the smearing procedure. Some concentrations of alpha tracks have been found unassociated with cells. The exact nature of this phenomenon is being investigated and will be reported at another time.

Two types of fog patterns interfering with interpretation can be pointed out in this photomicrograph. In the lower right-hand corner of the photograph is a fog streak. It is obviously different from the straight alpha tracks of one grain width. Neither can it be a beta track as it is too dense and straight. Nor does it fit the description of other nuclear particle tracks.⁶ This type of streak appears in the NTB stripping film and not in the NTB plate. We suggest that they originate in the stripping operation. In addition to the streaks the random fog grains should be noted. While these do not detract from the interpretation of an alphagraph, they do detract from that of a betagraph.

Stripping film to permit identification of histological bodies above sunbursts. Fig. 5-a and 5-b show alphagraphs which have been made by placing a liver section containing

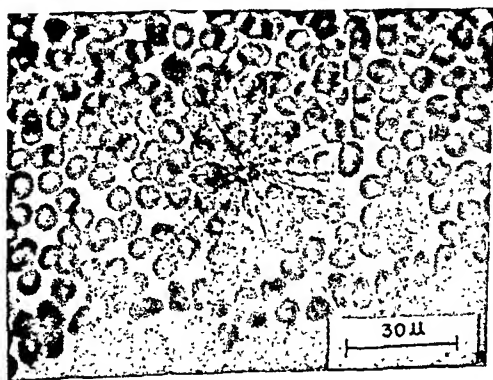


Fig. 4.

Sunburst of alpha tracks centered on a red cell.

⁶ Powell, C. F., Occhialini, G. P. S., *Nuclear Physics in Photographs*, Oxford at the Clarendon Press, 1947.

Comparison of the Preputial Glands in the Alexandrine, The Wild, and the Domestic Norway Rat.*

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During routine autopsies on wild Alexandrine and wild Norway rats, it appeared that the preputial glands of these wild rats were much larger than those found in the common domestic Norway rats. To check these impressions, the weights of the preputial glands were determined in the 3 forms for all sized rats from the smallest to the largest individuals.

Material and methods. For this study 78 domestic Norway, 121 wild Norway, and 90 Alexandrine rats were used.

The domestic Norways were obtained chiefly from our colony. These are descendants of Wistar rats established here over 20 years ago. The wild Norways were trapped in Baltimore streets and alleys and on surrounding farms by the Rodent Ecology Division of the Johns Hopkins School of Hygiene; the Alexandrines came from ships in the Baltimore harbor, where they had been killed by fumigation.‡

The domestic and wild Norways were killed with ether and autopsied immediately. The Alexandrines were not available for autopsy until 3 to 6 hours after they had been killed by fumigation.

At autopsy the rats were weighed; the preputial glands were removed and weighed on a torsion balance to the nearest 0.1 mg. In every case when sections were made, the glands were taken from recently killed animals and fixed in Bouin's solution with a minimum of handling.

Results. Gross anatomy: Fig. 1 shows a photograph of typical pairs of preputial glands taken from 3 female animals, a domestic Norway, a wild Norway, and a wild Alexandrine, all having about the same body weight. The glands from the wild Alexandrine are several times larger than those from the wild Norway and many times larger than those from the domestic Norway.

These glands are flattened, pear-shaped structures that lie between the skin and the lower abdominal musculature, one on either side of the penis or clitoris. Each gland terminates in a duct that emerges on the surface of the skin in a papilla-like opening. In the male, when the penis is fully retracted and thus ensheathed in the prepuce, the openings in the prepuce are located at the very end of the penis, whereas when the penis is fully erected and thus freed from the prepuce, the openings are located on the two sides at the base of the penis. In the female, the ducts emerge at the homologous points with relation to the clitoris.

A slight pressure exerted on the glands causes droplets of secretion to appear on the surface of the skin. These droplets help to locate the openings. In non-albinos, the presence of pigmentation in the walls of the ducts further helps to locate the openings.

Microscopic anatomy: A dense connective tissue capsule encloses the glandular parenchyma, which is composed of large, flat, polyhedral cells grouped into rounded acini and characterized by vesicular, pale-staining nuclei. Dissolution of the epithelial cells forms a secretion that appears as a granular eosinophilic mass within the numerous branched ducts.

Large wild Norway rats frequently have atypical glands in which numerous large cysts mar the usual smooth glandular surface (Fig. 2A). Such glands are found to consist of

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‡ Dr. M. F. Haralson, until recently in charge of the Quarantine Station of the City of Baltimore, kindly supplied these rats.

and 5-d were obtained. Only the low energy ends of the tracks are seen in 5-c, the concentration of silver grains being prevented by the insensitive layer of cellulose ester. Fig. 5-d shows sufficient illumination to observe and photograph the histological body directly above the center of the alpha track ends. This body (circled) appears to be a von Kupffer cell. This is a tentative conclusion awaiting further checking.

Stripping film to permit estimating relative amounts of beta emitters in tissue. The estimation of relative quantities of an alpha emitter is relatively easy since an alpha track representing a single disintegrated atom is a definite grain pattern and can easily be recognized. If the geometry and exposure time and half-life are known, a fair estimate of the concentration of an alpha-emitting element can be made. Certain precautions have to be taken. For example, the exposure must not be so long as to give a sunburst density as shown in Fig. 5-a in which the tracks cannot be counted. Also, if an optically dense histological body should override an alpha track, its chances of detection would be diminished.

These difficulties are magnified for the estimation of a beta emitter since no recognizable pattern of grains is produced. Instead, single grains must be counted. In the case of the thyroid, for example, where the colloid is reasonably transparent, the grains underneath the colloid can be easily distinguished one from the other. However, when one attempts to count the grains beneath the follicular epithelial cells, the grains are obscured by the optical density of the stained cells and their nuclei especially. This is illustrated in Fig. 3.

Stripping film offers an escape from this impasse. The tissue is mounted on the cellulose ester, 10 μ thick, instead of on the emulsion. Examining with a 1.8 mm objective having a field depth of about 0.5 μ and focusing the microscope 10 μ beneath a section to observe the grains, the optically dense bodies of the tissue are sufficiently out

of focus and thereby transparent to permit counting the grains immediately beneath them. However, this advantage is not gained without some loss in resolution due to the 10 μ separation of the tissue from the emulsion. But this is a small loss compared with the gain in resolution of the NTB emulsion over that of medium lantern slide emulsions.

An additional advantage of the stripping film technic is a method of laying a uniformly thin emulsion layer close to a single cell or tissue. Either the emulsion or the 10 μ base can be placed in contact with the specimen. The latter differs from the emulsion-painting technic of Bélanger and Leblond² in that the emulsion is separated from the tissue by the thickness of the base. The stripping film has the advantage over the painting technic in that the base and emulsion are standardized at the factory by those experienced in the art of making photographic emulsions.

Another advantage of the stripping film is its use in the prevention of chemical fogging. As emulsions become more sensitive to beta particles we will be able to detect smaller amounts of beta emitters in a tissue section. However, as the technic becomes more sensitive, the problem of eliminating fogging due to various naturally occurring chemicals in the tissue looms large. The mounting of sections on the cellulose ester base instead of directly on the emulsion will eliminate this difficulty.

Summary. An autoradiographic technic, using stripping film, is described by which back scattering, emulsion staining, chemical fogging, and photographic-developer damage to the tissue are eliminated; blood smear autographs can be made; histological bodies above intense collection of grain can be identified; and grain counting for quantitation is permitted.

We appreciate the valuable assistance of Mr. Robert Hay in the preparation of the final photographs for this paper.

This paper is based on work performed under contract with the United States Atomic Energy Commission at The University of Rochester Atomic Energy Project, Rochester, N. Y.

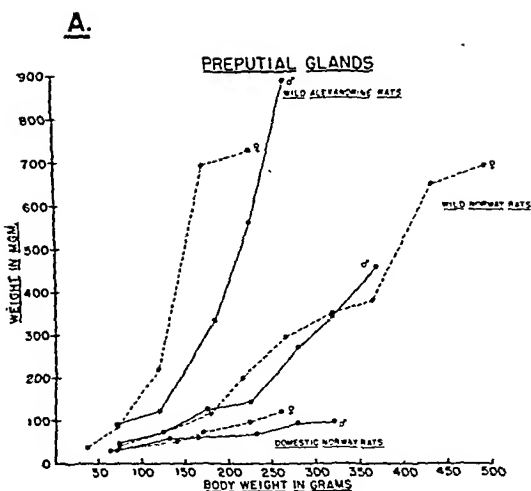


FIG. 3-A.

Graphs showing the relationship between the weights of the preputial glands and body weight in the 3 forms of rats: wild Alexandrines (45 ♂, 45 ♀); wild Norways (61 ♂, 60 ♀); domestic Norways (39 ♂, 39 ♀).

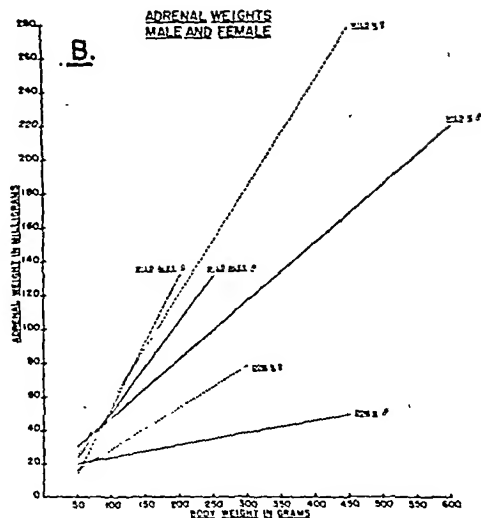


FIG. 3-B.

Graphs showing the relationship between the weights of the adrenals and body weight in the 3 forms of rats. (Data taken from Rogers and Richter).

Physical characteristics of preputial secretion: A considerable quantity (0.1-0.3 cc) of the sebaceous secretion usually may be expressed from the ducts of the glands by gentle manipulation in the mature wild Norway and the Alexandrine rats. It is seldom possible, however, to obtain as much from the glands of the domestic rat. In many rats the secretion is a viscous yellow mass; in some it may be quite liquid. In the former case it emerges in ribbon form, whereas in the latter droplets are formed.

Growth and size of preputial glands: Fig. 3A shows the relation between the weight of the preputial glands (in mg) and body weight. The lines connect points representing average values obtained in the various body weight classes.

a) *Wild Norways*. The weights of the preputial glands in female rats increased from 30 mg at an average body weight of 75 g to 695 mg at 500 g, or a 23-fold increase. The gland weights of the males increased at much the same rate.

b) *Domestic Norways*. The glands weighed much less in all body-weight classes of the domestic rats than in the wild forms, and

their size increased at a much less rapid rate. The weights of the glands of female rats increased from 25 mg at an average body weight of 60 g to 115 mg at 350 g, or a 4.5-fold increase. Over the same weight range the glands of the wild Norways increased 11-fold. In general, female rats had larger preputial glands than males of the same size.

c) *Alexandrine rats*. At body weights of 250 g, the glands averaged 760 mg for Alexandrines, 200 mg for wild Norways, and 90 mg for domestic Norways. Thus the glands of Alexandrines weighed about 4 times as much as the glands of wild Norways and about 8.5 times as much as those of domestic Norways.

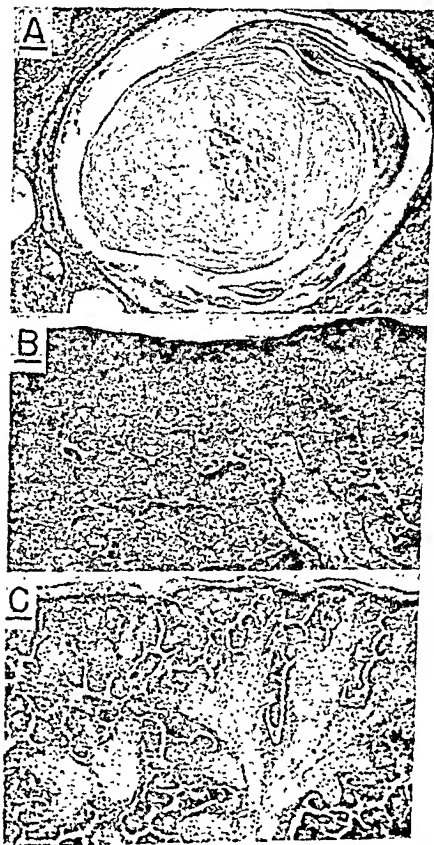
A comparison of the heaviest glands found at any time in each species further brings out the species differences: 143 mg in the domestic rat, 984 mg in the wild Norway, and 1200 mg in the wild Alexandrine.

The sharp increase that occurs in the size of the glands in the wild Alexandrine rat at body weights between 75 and 125 g, and in the wild Norways between 175 and 250 g, may coincide with the onset of puberty in the two species.



FIG. 1.

Photograph of the 3 pairs of preputial glands from a domestic Norway, a wild Norway, and a wild Alexandrine rat. Each rat weighed 250 g. (Scale—centimeters).



numerous dilated ducts, separated by strands of connective tissue. Many of these ducts show hyperkeratosis. Such cysts are generally associated with a chronic inflammatory reaction. It is not clear whether the inflammation causes, accompanies or results from the epithelial cysts.

Apart from this abnormality, routine staining with hematoxylin and eosin does not reveal any essential difference in glandular structure between the 3 types of rats. Figs. 2B and C are photomicrographs which show the typical structure of the preputial glands in Alexandrine and domestic Norway rats.

FIG. 2-A.

Low power view ($\times 100$) of a section from a preputial gland from a wild Norway rat. The center of the field is occupied by a large cystic dilatation containing a typical laminated epithelial "pearl" in the center of which degenerating acini may be seen.

FIG. 2-B.

Low power view ($\times 100$) of a section from the preputial gland of a domestic Norway rat. A branch of one of the ducts extends upwards from the lower right hand corner.

FIG. 2-C.

Low power view ($\times 100$) of a section of the preputial gland from a wild Alexandrine rat. A large multibranched duct is the most prominent feature.

size. In the cross-bred offspring, the glands are markedly smaller than those in wild Norway rats and only slightly larger than those in domestic Norways.

Relation of preputial glands to adrenals: Fig. 3B gives the average adrenal growth curves obtained for 100 (50 ♂ and 50 ♀) each of wild Alexandrine, wild Norway, and domestic Norway rats (data redrawn from Rogers and Richter⁵). The ordinates give the combined weights of both adrenals in milligrams; and abscissas, body weight in grams. Like the preputial glands, the adrenals of the wild rats are much larger than those of the domestic rats, and in each form the females have the larger glands. Unlike the preputial glands, however, the adrenals are only slightly larger in the wild Alexandrines than in the wild Norways.

In crosses between wild and domestic Norway rats the adrenals, like the preputial glands, are only slightly larger than in the domestic forms.

Discussion. The common laboratory Norway rat has undergone great changes in behavior during the course of domestication. It is tame, tractable and trusting; the wild rat is fierce, intractable, and suspicious. Some of the behavior changes can be correlated with anatomical modifications, such as the decrease in size of the adrenal glands (Rogers and Richter,⁵) or the decrease in the number of taste papillae on the surface of the tongue (Fish and Richter⁶). No such correlation can be readily detected between behavior and the

recession of the preputial glands. The lack of any definite knowledge about the function of these glands makes a correlation difficult.

Since the ducts in males are located on the penis, it is possible that this highly sebaceous secretion may serve a lubricating function during copulation (Schaffer⁷). It seems unlikely, however, that it serves such a function in females, since in them the ducts do not end near the vaginal opening.

The secretion, through its odor (Noble and Collip¹), might play a part in sex attraction. The fact that domestic rats have lived in cages for such a long time may be related in some way to the atrophy of these glands. Mates are provided for domestic rats, so that they no longer have to depend on finding their mates by scent.

The larger size of the preputial glands in the wild Alexandrines remains unexplained. The Alexandrine rats live more in ceilings, where any scent left would not be destroyed by rains, etc., as it is on the ground. Perhaps scent plays a larger part in their life than in that of the wild Norways.

Summary. 1. The domestic Norway rat, the animal commonly used in laboratories throughout the world, has much smaller preputial glands than does its wild ancestor.

2. The preputial glands are definitely larger in the wild Alexandrine than in the wild Norway.

3. In all 3 forms, the glands are larger in females than in males.

4. The preputial glands and the adrenals seem to show the same growth relationships in the three forms.

⁵ Rogers, P. V., and Richter, C. P., *Endocrinology*, 1948, 42, 46.

⁶ Fish, H. S., and Richter, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1946, 63, 352.

⁷ Schaffer, J., *Z. mikr.-anat. Forsch.*, 1933, 34, 1.

In the domestic Norway, which always has small glands, the variations from the mean for any weight group are small, whereas in the other two forms which have large glands, there is much more scattering, especially in the largest animals.

While comparing the size of the preputial glands in the three forms, one should bear in mind that the Alexandrine rat is normally a smaller animal than the Norway. A 250 g Alexandrine is an old adult of maximal size, but a 250 g wild Norway is barely an adult. If a comparison could be made of the relationship between preputial gland size and age, the difference between Alexandrine and wild Norway rats probably would not be as great as indicated by the present method. Unfortunately it is not possible to determine the age of trapped wild rats accurately, and laboratory raised Alexandrines of known age have not been available.

The preputial glands of both wild Norway rats and Alexandrine rats attain a greater size than has ever been produced experimentally with any form of treatment in the domestic Norway. Glands weighing about 300 mg have been reported in domestic rats following treatment either with potent adrenocorticotrophic extracts of the hypophysis (Noble and Collip,¹) or androgens (Korenchevsky, *et al.*,²⁻⁴) but these are of moderate size compared to those found normally in adult Alexandrine or wild Norway rats.

Cross breeding: The offspring of both sexes from a cross between a wild Norway male and a domestic Norway female were used to determine the effects of cross breeding on the size of the preputial glands. Table I shows the relationship between the size of the preputial glands and body weight in the offspring of this cross as compared with wild Norway and domestic Norway rats of equal

TABLE I.
Weights of Adrenal and Preputial Glands in Wild and Domestic Norway Rats and in First Generation Crosses.

Type	No. of females	Body wt g	Preputial glands mg	Adrenals mg	No. of males	Body wt g	Preputial glands mg	Adrenals mg
Wild Norway	7	215 (200-245)	254 (144-335)	90.1 (58.3-120.0)	6	358 (330-370)	426 (243-708)	103.6 (74.5-158.0)
Wild x Domestic (F ₁)	7	246 (205-229)	111 (90-136)	66.5 (47.1-80.1)	6	355 (327-369)	126 (94-168)	46.8 (45.0-48.2)
Domestic Norway	7	220 (212-224)	108 (72-127)	52.9 (41.3-67.6)	4	355 (352-359)	109 (100-115)	27.8 (20.6-35.3)

¹ Noble, R. L., and Collip, J. B., *Endocrinology*, 1941, 29, 934, 943.

² Korenchevsky, V., Dennison, M., and Simpson, S. L., *Biochem. J.*, 1935, 29, 2534.

³ Korenchevsky, V., Dennison, M., and Eldridge, M., *Biochem. J.*, 1937, 31, 467, 780.

⁴ Korenchevsky, V., Hall, K., and Burbank, R., *Biochem. J.*, 1939, 33, 213, 372.

ginning of this project, and no treatment other than the drugs being tested was given until after this work was completed. All subjects had received the routine hospital studies at the time of admission. Recent additional tests, including chest x-ray, E.C.G., C.B.C. and urinalysis, were within normal limits.

Evaluation of the subjects at the time of administration of the drugs included determinations of blood pressure, pulse rate, skin temperature, sweat secretion, electrical skin resistance, and neurologic and psychiatric status.

Arterial blood pressure was measured in one arm by the standard auscultatory method. Radial pulse rate was counted for 15 seconds.

Skin temperature was measured using a Hardy Dermal Radiometer. Readings were taken from the forehead and from the palm and dorsum of the hand contralateral to the arm in which the infusion was given. Room temperature was also recorded.

Permanent records of palmar sweat secretion were made according to the technic described by Silverman and Powell.⁷ Diluted tincture of ferric chloride was painted on the dried, free palm and allowed to evaporate. A print was obtained by holding the palm for 3 min. against a sheet of paper impregnated with tannic acid. Thus the secretory activity of each sweat gland was recorded by a dot of ferric tannate. The number, size and distribution of these dots could be noted readily, and the amount of sweating could be rated according to previously described standards.⁸ A faint response (0) included prints varying from almost entirely blank to those showing a light grey shade with fine pinpoint dots. A moderate response (1+) showed thicker dots, darker in shade, arranged in a linear pattern. A strong response (2+) showed black speckles of varying sizes. An intense response (3+) showed blackening with blotches.

Electrical skin resistance was measured using a dermohmometer of the Jasper type.⁹ The

electrodes were pure silver discs, 1 cm in diameter. No electrolyte paste or jelly was used at either electrode. The resistance was measured between pads of the hallux and the index-finger of the same hand, giving a single reading per patient per examination. This thumb to finger resistance was quickly and simply obtained, and remained constant at an examination. It represents approximately double the value which is obtained if electrolyte paste or jelly is used to reduce the resistance at one electrode.

All neurologic examinations and evaluations of the signs of catatonia were made by the same observer and in a uniform manner. Twelve categories in which abnormalities are frequently found in catatonics had been previously selected. These categories were: posturing, facial immobility, facial grimacing, spontaneous activity, spontaneous speech, activity in response to request, speech in response to questions, strength of manual grip, passive resistance, waxy flexibility, response to pin-prick, and response to supraorbital pressure. In order that comparisons could be made, each subject was rated from 0 to 4+ in each of the 12 categories, according to the amount of deviation from the normal. The results of previous tests were not consulted at the time of subsequent examinations. Consistency of the evaluations was readily demonstrated.

Psychiatric examination consisted of observations and interviews lasting about 30 minutes per patient. All were done by the same examiner, who was already acquainted with the subjects and their histories. Detailed notes were kept.

The drugs employed were sterile sodium amytal, supplied in 0.5 g ampules, and sterile Dibenamine, supplied in ampules containing 500 mg of the drug in 10 cc acidified alcohol-propylene glycol. All drugs were administered intravenously in sterile, isotonic saline solution, supplied in 500 cc flasks, as described below.

Procedure. The investigation was carried out in an ordinary 12-bed hospital ward as-

⁷ Silverman, J. J., and Powell, V. E., *Am. J. M. Sc.*, 1944, 208, 297.

⁸ Silverman, J. J., and Powell, V. E., *Psychosomatic Med.*, 1944, 6, 244.

⁹ Jasper, H., *J. Neurosurg.*, 1945, 2, 257.

Effect of N,N-Dibenzyl- β -Chloroethylamine Hydrochloride (Dibenamine) on Autonomic Functions and Catatonia in Schizophrenic Subjects.*

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The schizophrenic reaction, catatonic type,¹ (dementia praecox, catatonic²) is characterized by conspicuous abnormalities of motor behavior. Patients with this disorder frequently also show signs of imbalance of the autonomic nervous system. Pupillary changes, vasomotor disturbances, increased salivation, scanty or increased urine, constipation, and cardiovascular irregularities may occur.³ Gellhorn concluded that studies of autonomic functions in schizophrenic patients demonstrate "not only a decreased reactivity of the sympathico-adrenal system, but also a relative preponderance of the vago-insulin system."⁴ Nevertheless, mydriasis, delayed gastric and colonic emptying,⁵ cold extremities, and hyperhidrosis indicate elements of increased sympathetic activity.

The object of this investigation was to study the effect of chemical blockade of sympathetic function in patients with schizophrenic reactions, catatonic type. The drug used was Dibenamine (N,N-dibenzyl- β -chloroethylamine hydrochloride),[†] a synthetic ter-

tiary amine having the specific, powerful and prolonged action of blocking excitatory adrenergic activity.⁶

In October 1947, as a preliminary procedure, Dibenamine was given to 2 catatonic patients at the New Jersey State Hospital at Greystone Park. Definite changes in autonomic functions and transient improvement in the catatonia were observed. Controlled studies were therefore arranged. The present report concerns our investigation of a group of catatonic subjects in whom certain autonomic functions were evaluated and neurologic and psychiatric examinations were made before and after administration of saline, sodium amytal and Dibenamine infusions.

Methods and Materials. Nine subjects, diagnosed as having schizophrenic reactions, catatonic type, were selected from the general wards at the Veterans Administration Hospital, Lyons, N. J. Patients with marked impairment of spontaneous activity and poor responsiveness to stimulation were sought. The cases with the severest stigmata of catatonia were chosen. The ages of the subjects ranged from 22 to 38 years, and their psychoses had been present from 3 to 7 years. Subject 4 was the only one who showed any recent tendency to have spontaneous remissions. Most of the patients had received electric and/or insulin shock therapy, with which varying degrees of transient benefit had been noted. No shock treatment was given to any of the group for at least 10 weeks prior to the be-

* Published with permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the authors.

¹ Nomenclature of Psychiatric Disorders and Reactions, Vet. Adm. T.B. 10A-78, Oct. 1, 1947.

² Hinsie, L. E., and Shatzky, J., *Psychiatric Dictionary*; London, New York, Toronto, Oxford University Press, 1940.

³ White, W. A., *Outlines of Psychiatry*, Washington, Nervous and Dental Disease Publishing Co., 1935.

⁴ Gellhorn, E., *Autonomic Regulations*, New York, Interscience Publishers, Inc., 1943.

⁵ Henry, G. W., *Am. J. Psychiat.*, 1928, 7, 135.

[†] Dibenamine supplied through the courtesy of Dr. W. Gump, Givaudan-Delawanna, Inc., Delawanna, N. J.

⁶ Nickerson, M., and Goodman, L. S., *J. Pharm. and Exp. Therap.*, 1947, 80, 167.

TABLE I.
Effect of Dibenamine on Blood Pressure and Pulse Rate in Catatonic and in Normotensive, Non-Psychotic Subjects.

	Catatronics	Non-psychotic normotensives ¹⁰
Resting B.P.	115 \pm 1.4	124 \pm 2.2
	76 \pm 1.2	73 \pm 1.7
B.P. after Dibenamine	121 \pm 3.3	122 \pm 3.0
	67 \pm 1.8	69 \pm 1.9
Change in B.P.	+6 \pm 3.1	-2 \pm 2.6
	-8.5 \pm 1.0	-4 \pm 1.5
Resting P.R.	73 \pm 2.9	78 \pm 2.0
P.R. after Dibenamine	110 \pm 6.1	84 \pm 2.8
Change in P.R.	+37 \pm 8.8	+6 \pm 1.6

tachycardia lasted 18 to 36 hours. The morning after the infusions the mean P.R. was 96 ± 6.8 , with 2 of the 9 subjects who had received 5 mg/kg and 2 of the 3 who had received 7.5 mg/kg still having resting rates over 100. All subjects had orthostatic tachycardia when tested 18 hours after the Dibenamine infusions. This lasted 24 to 72 hours.

It is of interest to compare the B.P. and P.R. effects of Dibenamine in this group of catatonic subjects with previously reported results in non-psychotic, normotensive general hospital patients.¹⁰ (Table I.) The methods and circumstances of both studies were similar. The initial systolic pressure is significantly lower in the catatonics (difference in means = 9 ± 2.6), although there is no significant difference in diastolic pressures. B.P. studies in a larger series showed significantly lower systolic and diastolic pressures in schizophrenic patients compared with students.¹¹ Following Dibenamine the mean B.P. of the catatonic group is almost identical with that of the non-psychotics. However, although the initial P.R.s in the two groups did not differ significantly, the catatonics showed a very much faster P.R. fol-

lowing Dibenamine.

The response to Dibenamine of catatonic subjects differed from that of non-psychotic normotensives by showing a slight increase in systolic pressure and a large increase in P.R. These differences are suggestive of 2 effects of injected epinephrine which are not blocked by Dibenamine, namely the rise in cardiac output¹² and the tachycardia.^{6,12,13} The presence or production of increased epinephrine in the catatonics might explain the observed differences. Further speculation will be deferred until additional studies are completed.

(C) *Skin Temperature.* Forehead, palm, hand dorsum and room temperatures were recorded. Room temperature varied between 20.0° and 22.4°. Although even this small amount of variation was undesirable, it did not appear to affect the skin temperature results significantly. Furthermore, since all three drugs were being administered to the groups of subjects at the same time, comparison of the mean changes of the groups is valid.

The mean pre-injection forehead temperature was $34.1 \pm .2^\circ$, dorsum temperature was $27.4 \pm .5^\circ$, and palm temperature was $27.4 \pm .6^\circ$. No drug produced significant changes in forehead temperatures. Following saline infusions the hands were slightly cooler in all subjects. The mean change was $-1.7 \pm .5^\circ$ for the dorsum and $-2.5 \pm .8^\circ$ for the palm. This cooling probably represents the effect of the additional 1 to 1½ hours of exposure with relative immobility. After sodium amytal the mean dorsum temperature change was $+3.2 \pm 1.5^\circ$ and the mean palm change was $+3.0 \pm 1.4^\circ$. These mean temperatures (increases) differ from those following saline (decreases) by $4.9 \pm 1.6^\circ$ and $5.5 \pm 1.6^\circ$, both significant differences. This warming of the hands after sodium amytal was noted in 5 of the 6 subjects who were

¹² Hecht, H. H., and Anderson, R. B., *Am. J. Med.*, 1947, 3, 3.

¹³ (a) Acheson, G. H., Farah, A., and French, G. N., *Fed. Proc.*, 1947, 6, 305. (b) Youmans, W. B., and Rankin, V. M., *Proc. Soc. Exp. Biol. and Med.*, 1947, 66, 241.

¹⁰ Haimovici, H., and Medinets, H. E., *Proc. Soc. Exp. Biol. and Med.*, 1948, 67, 163.

¹¹ Freeman, H., Hoskins, R. G., and Sleeper, F. H., *Arch. Neurol. and Psychiat.*, 1932, 27, 333.

signed for the purpose. The subjects were kept lightly restrained in bed with their hands exposed for 2 hours preceding the testing, which was begun at about 9:00 a.m. Breakfast and lunch were withheld. After pre-injection observation (blood pressure, pulse rate, skin temperature, palmar sweat secretion, electrical skin resistance, neurologic and psychiatric status), intravenous infusions were started in all subjects, with 400 cc saline in the flasks. When the infusions were running well, 0.5 g of sodium amytal was added to the saline in each of 3 flasks, and mixed well. Similarly, Dibenamine in dosage of 5 mg per kg of body weight of the subject was added to the saline in each of 3 other flasks, and mixed well. The remaining 3 subjects received only saline. The infusion rate was adjusted to approximately 100 drops per minute, so that 1 to 1½ hours were taken for administration of the entire volume. In 4 subjects, whose reactions were inadequate after 350 cc of the dilute sodium amytal solution had been given, additional sodium amytal (up to 0.5 g) was slowly injected directly into the infusion tubing. At the end of each Dibenamine infusion, the tubing and vein were flushed by the addition of about 75 cc of saline to the flask. Immediately following the infusions the pre-injection tests and examinations were repeated. The subjects were kept in bed, but beginning that evening regular meals were served. After breakfast the next morning the tests and examinations were given for the third time, and those subjects who had received saline or amytal were allowed up ad lib. Those who had received Dibenamine were kept in bed until the orthostatic hypotension and tachycardia were gone (24 to 72 hours).

The first series of infusions was given March 9, 1948. Two weeks later the entire procedure was repeated, but the subjects were rotated with respect to the drug received. Two weeks after the second series of infusions, the third series was given, with the subjects again rotated. Thus, each patient received 3 infusions—one saline, one saline and sodium amytal, and one saline and Dibenamine (5 mg/kg body weight). (The only exception

to this protocol was Subject 6, who was not given his third infusion, saline, because his severe feeding problem made it necessary to institute electric shock therapy.) The fourth and final series of infusions was given 2 weeks after the third. Three subjects who had previously responded comparatively poorly to Dibenamine were selected. This time they received Dibenamine in dosage of 7.5 mg per kg body weight (maximum dose was 500 mg), with the remainder of the procedure for them carried out as on the previous occasions.

Results. (A) Blood Pressure (B.P.): Pre-injection resting B.P. ranged from 124/80 to 96/68, with a mean of $115 \pm 1.4/76 \pm 1.2$.† Neither saline nor sodium amytal resulted in significant B.P. changes, the post-injections means having been $109 \pm 2.9/72 \pm 1.5$ and $110 \pm 3.6/71 \pm 3.1$ respectively. The mean B.P. following the 12 Dibenamine infusions was $121 \pm 3.3/67 \pm 1.8$. Although 4 of the Dibenamine infusions were followed by rises in systolic pressure averaging 15 mm each, the increase in the mean is not statistically significant. The mean change in the diastolic pressure was a significant decrease of 8.5 ± 1.0 , representing the effect of blockade of sympathetic vasoconstrictor tone. Eighteen hours after Dibenamine administration the mean B.P. was $121 \pm 2.7/68 \pm 2.2$, and cardiovascular effects of the drug were still in evidence. No testing for orthostatic hypotension was done immediately after the Dibenamine infusions. However, the following morning this phenomenon was noted in 3 of the 9 subjects who had received 5 mg/kg and in all 3 who had received 7.5 mg/kg. Orthostatic hypotension had disappeared from all subjects by the third post-injection day.

(B) Pulse Rate (P.R.): Pre-injection resting P.R. ranged from 60 to 96, with a mean of 73 ± 2.9 /min. The saline and the sodium amytal infusions produced no significant alterations in the P.R. Following the 12 Dibenamine infusions the mean resting P.R. was 110 ± 6.1 , a mean increase of 37 ± 6.8 over the pre-injection levels. This

† All means are given \pm the standard error of the mean.

patients in catatonic stupor was found to be about the same as those of normals and of other groups of psychotic subjects.¹⁵ A relation between a large amount of "free energy (or anxiety)" and low E.S.R., and between a small amount of free energy and high E.S.R. has been suggested.¹⁶

One may speculate regarding the mechanism by which Dibenamine inhibited excessive sweating and raised low E.S.Rs. in the subjects who showed these pre-injection abnormalities. The regions tested (palms and finger pads) have sweating responses which are of the emotional type, specific for conditions of mental stress.¹⁷ Dibenamine therefore may have produced the observed changes indirectly, as a result of a central action in eliminating an "anxiety" factor. Against this explanation is the failure of sodium amytal to have produced a comparable autonomic effect. The second possibility is that Dibenamine acted on sympathetic ganglia or directly on the sweat glands. These would imply either that Dibenamine inhibited a cholinergic function,¹⁸ or that the excessive sweating and low E.S.Rs. were exhibitions of adrenergic activity. There is no evidence to suggest that Dibenamine is anti-cholinergic. However, there is some reason to believe that certain sweating may have an adrenergic component.¹⁹ It is also possible that Dibenamine decreased the sweating and increased the low E.S.Rs. by an action upon the hypothalamic sympathetic mechanism. Although there is no present direct evidence for this explanation, it is in accordance with other features of Dibenamine's central activity, which are described below.

(F) *Neurologic Signs and Signs of Catatonia.* Except for the physical signs of cata-

tonia described below, no subject showed an abnormal neurologic status. Deep tendon reflexes ranged from hypo-active to hyper-active, with 3 tense subjects having occasional transient clonus. Pupils were equal bilaterally in all subjects, and one showed mydriasis. None of the drugs administered changed the general neurologic status significantly. However, transient clonus was not observed after sodium amytal or Dibenamine, and all subjects had miosis following Dibenamine.

As previously outlined, the signs of catatonia were observed in each subject and the amount of abnormality present in each of 12 categories was rated from 0 to 4+. It was therefore possible to record not only general impressions of improvement in the catatonia but also changes in each of the categories. In addition, the total number of points of abnormality shown by a subject at an examination may be considered his "catatonic rating" at that time, and used to rank the subject with regard to severity of the catatonic manifestations.

Pre-injection examinations revealed catatonic ratings for the individuals ranging from 13 to 40, with a mean of 26.5 ± 1.3 . The following summarizes the pre-injection findings.

All subjects appeared to be content to lie lightly restrained in bed. Abnormal posturing was common, and was present to a marked degree in 3 subjects. Blankness of facial expression was the rule, although 2 subjects usually showed meaningless grimacing and 3 others grimaced occasionally. Spontaneous motor activity was rare in any of the group. No subject except No. 5 (on one occasion) evinced spontaneous speech during pre-injection testing. Subjects 4 and 5 were the only ones who would respond, even in monosyllables, when questioned prior to the injections. When requested or commanded to perform simple motor actions (lift arm, close eyes, open mouth), 4 subjects rarely or never complied, although 2 others were usually completely cooperative. No subject demonstrated more than feeble effort or strength of manual grip when requested, but considerable force was used by 3 subjects to resist certain pas-

¹⁵ Syz, H., and Kinder, E., *Arch. Neurol. and Psychiat.*, 1928, 19, 1026.

¹⁶ (a) Solomon, A. P., and Fentress, T. L., *J. Nerv. and Ment. Dis.*, 1934, 80, 163. (b) Darrow, C. W., and Solomon, A. P., *Arch. Neurol. and Psychiat.*, 1934, 32, 273.

¹⁷ Kuno, Y., *Lancet*, 1930, 1, 912.

¹⁸ (a) Feldberg, W., and Gaddum, J. H., *J. Physiol.*, 1934, 81, 305. (b) Dale, H. H., and Feldberg, W., *J. Physiol.*, 1934, 82, 121.

¹⁹ Haimovici, H., *Proc. Soc. Exp. Biol. and Med.*, 1948, 68, 40.

neurologically improved by the drug, and in 1 of the 3 who were not. Following Dibenamine the mean change in dorsum temperature was $+2.9 \pm 1.3^\circ$ and the mean change in palm temperature was $+3.6 \pm 1.2^\circ$. These differ from the mean changes after saline by $4.6 \pm 1.4^\circ$ and $6.1 \pm 1.4^\circ$, both significant differences. The increases in hand temperature after Dibenamine ranged up to 10.3° , and the greater changes were noted in the subjects who were more improved neurologically by the drug. Eighteen hours after Dibenamine infusions the increase in hand temperature was still present, the mean changes for the dorsum and palm being $+4.6 \pm .7^\circ$ and $+5.4 \pm .8^\circ$ respectively. Eighteen hours after the saline and the sodium amytal infusions the hand temperatures were not significantly different from pre-injection values.

(D) *Sweat Function*: Twenty-seven pre-injection palmar sweat prints were taken from 8 subjects. (No prints were obtained from Subject 6 because his pre-injection posturing included tightly clenched fists.) A faint response was found in 15 (55%) pre-injection prints, a moderate response in 6 (22%), a strong response in 2 (8%), and an intense response in 4 (15%). Individual variation between the days of examination was noted. However, 4 subjects usually produced almost entirely blank prints, whereas 2 others had hyperhidrosis consistently and 1 other had hyperhidrosis occasionally. Neither saline nor sodium amytal infusions produced significant changes in the sweating responses, but immediately following the Dibenamine infusions there were no longer any strong (2+) or intense (3+) responses. Of the 11 prints obtained after Dibenamine, 10 were faint (0) responses and 1 was a moderate (1+) response. This inhibition of excessive palmar sweating gradually disappeared. Eighteen hours after injection, Subject 3 again had an intense (3+) response and Subject 9 had a moderate (1+) response.

Palmar sweat prints obtained by the same technic and rated according to the same standards as the prints in this project were reported to have shown faint or moderate re-

sponses in 78% of a group of hospital personnel and strong or intense responses in 84% of the neuropsychiatric (mostly psychoneurotic) patients.

(E) *Electrical Skin Resistance (E.S.R.)*: The mean pre-injection E.S.R. for all infusions in the 9 subjects was 192 ± 18 K.[§] Three subjects, the same ones who had excessive sweating, showed E.S.R. values clearly abnormally low for the testing conditions. The mean pre-injection E.S.R. for these 3 was 105 ± 21 K, whereas for the 6 other subjects the mean was 238 ± 17 K. Five subjects showed pre-injection E.S.R. values which were constant for the several series of tests, but in the other 4 there were large spontaneous day to day variations. Neither saline nor sodium amytal infusions were followed by significant changes in the E.S.R. Immediately after the Dibenamine, the mean E.S.R. was 258 ± 25 K, an increase of 66 ± 30 K from the pre-injection mean; 18 hours post-injection the mean was 290 ± 17 K, an increase of 98 ± 24 K. The increases in E.S.R. after Dibenamine were noted to occur almost exclusively in those subjects whose initial resistances were low. Thus for 8 infusions in 6 subjects whose pre-injection values were under 200 K, the mean change immediately following Dibenamine was $+151 \pm 40$ K and 18 hours later it was $+195 \pm 40$ K.

The palmar sweat responses and the E.S.R. measurements made in this study and summarized above show a very high correlation. (Spearman method of rank: $r = .48$, $t = 4.4$, and $p = \text{less than } .001$.)

The initial observations were made with the subjects at basal conditions. The very low sweat responses with high E.S.Rs. found in 4 subjects in contrast with the hyperhidrosis and very low E.S.Rs. found in 3 others demonstrate the extremes of these functions which catatonic patients may show. Wide variation in the E.S.Rs. of catatonics may be found in the data of other reports,¹⁴ although the average palm-to-palm E.S.R. of a group of

[§] K. = 1000 ohms.

¹⁴ (a) Richter, C. P., *Arch. Neurol. and Psychiat.*, 1928, 19, 488. (b) Richter, C. P., *Arch. Neurol. and Psychiat.*, 1929, 21, 363.

noid) symptoms previously not elicitable were observed. When the catatonia was dissolved, there was evidence of ego-centered and introspective concern, with little interest in the surroundings. At a later stage 4 subjects were moderately sleepy, and 1 was deeply so. Dibenamine, in contrast, produced definitely increased interest in surroundings, increased spontaneity, improved responsiveness to environmental stimuli, and no sleepiness. As with sodium amytal, every patient who had speech function after Dibenamine gave expression to hebephrenic or paranoid mental content.

The mechanism by which Dibenamine produced these changes in the catatonia may be considered. In non-psychotic individuals central effects have been observed after Dibenamine.^{10,12} Drowsiness is common, and mental confusion with restlessness, or irritability, emotional lability, hallucinations, paramnesia or perseverations have been noted in some patients. Convulsions can be produced by rapid administration in animals⁶ and were reported to have occurred in one patient.¹² Nickerson and Goodman state that the central stimulant action of Dibenamine is quite unrelated in time and mechanism to the adrenergic blocking activity, and that almost identical central effects can be produced by the hydrolysis product, N,N-dibenzylethanolamine, which has no adrenergic blocking action.²⁰ Investigation of this and other related compounds should be illuminating. Malononitrile, $\text{CH}_2(\text{CN})_2$, has been reported to produce an effect on psychic functions in mental disorders and to stimulate nucleoprotein production in nerve cells.²¹ Although flushing of the face resulted from malononitrile administration, there was no observed change in B.P. The cyanide radical can cause circulatory changes and has been shown to produce vasodilation, probably by paralysis of smooth muscle in vessel walls.²² Catatonia has

been observed to dissolve upon I.V. administration of 1/50 N. sodium cyanide solution.²³ While Dibenamine may have a direct effect upon nerve cell metabolism, it is not impossible that the improvement in the catatonia which followed its use also may be associated with alterations in cerebral circulation, particularly in the region of basal arterial supply. The correlation between neurologic change and change in skin temperature suggests at least parallel development of the increase in peripheral circulation and the improvement in the catatonia. The most recent study does not support the contention that the total cerebral blood flow in schizophrenics is abnormal.²⁴ The effect of Dibenamine on the circulation of blood in the brain of animals is being studied.

In this investigation, the responses to Dibenamine and the responses to sodium amytal were different. Only one of the 4 subjects who were greatly benefited by sodium amytal was among the 4 who were most improved by Dibenamine. Furthermore, the types of responses produced by the two drugs were characteristically dissimilar, the amytal changes being especially verbal and responsive and the Dibenamine changes being increased spontaneity and interest in surroundings. It would not appear that the mechanisms of action of these drugs are the same.

(H) *Toxic and Side Effects.* Few of the previously reported toxic effects of Dibenamine^{10,12} were observed in these subjects. There were no instances of phlebotrombosis, although Subject 4 complained of pain along the vein throughout the infusion. Only one subject appeared to be nauseated or vomited (No. 3, who had brief retching after each of the 2 Dibenamine infusions given him. He was an intermittent soiler, and also showed loss of sphincter control following one administration of Dibenamine). Increased drowsiness was not produced. When Subject 2 exhibited masturbatory activity after Dibenamine (4th series of infusions), he and the 2 other patients who had received the

²⁰ Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1948, 7, 397.

²¹ Hyden, H., and Hartelius, H., *Acta Psychiat. et Neurol.*, 1948, 48, 1.

²² Solhmann, T., *A Manual of Pharmacology*, New York, W. B. Saunders, 1942.

²³ Lorenz, W. F., *Psychiat. Quart.*, 1930, 4, 95.

²⁴ Kety, S. S., et al., *Am. J. Psychiat.*, 1948, 104, 765.

sive movements. All but 3 subjects occasionally showed variable degrees of waxy flexibility, but none would hold an induced posture for more than several seconds. The responsiveness to pin-prick and to supra-orbital pressure was markedly diminished or absent in 4 subjects, and in 2 others it was increased to a startle reaction.

Immediately following the saline infusions no subject was significantly changed. The number of points change for each subject ranged from +3 to -3, with a mean of $.3 \pm .9$ points improvement. Eighteen hours later the mean change was $.4 \pm 1.3$ points improvement. Following amytal, 4 of the 9 subjects were improved 11 to 19 points each, and the mean change was 6.6 ± 2.7 points improvement. The most striking result of the amytal infusions was the production of fair to good verbal responsiveness in 4 subjects. There was also considerably less posturing and no grimacing. Spontaneous activity and spontaneous speech remained poor in all except No. 6, who developed marked resistance in addition. Most subjects were generally relaxed by the drug, and no improvement in strength occurred. Responses to noxious stimuli approached normal in 6 subjects, but were less than normal in 3. The improvement which the sodium amytal produced lasted up to 4 hours, except in Subject 6, who was still improved (7 points) the next day. Eighteen hours after sodium amytal infusions, the mean change from the pre-injection status was 1.1 ± 1.1 points improvement.

Following the 12 Dibenamine infusions, the 9 subjects showed improvements ranging from 0 to 19 points each, with the mean change being 9.2 ± 2.5 points improvement. Nine of the 12 infusions resulted in improvements of 8 points or more, and in these subjects the change in status was quite apparent. Posturing persisted in only one subject of the entire group, and grimacing in but one other. Spontaneous activity was clearly increased in 6 instances, with spontaneous speech appearing in 3 to a considerable degree. Five subjects responded well to requests for simple motor activity and 4 had good speech response.

Strength of manual grip did not improve significantly. Although Subject 3 developed resistance to passive motions, all 6 subjects showing such resistance prior to drug administration were improved. Waxy flexibility decreased significantly in Subject 1. Three subjects developed more normal responses to pin-prick and to supra-orbital pressure. The improvement following Dibenamine gradually disappeared in most subjects over a period of 24 to 48 hours. Eighteen hours after the infusions, the mean change from the pre-injection status was 5.8 ± 1.7 points improvement. Speech function was the earliest to regress, disappearing a few hours after the infusion. (Subject 4 had a markedly atypical response to Dibenamine. His improvement began 5 minutes after the drug was started, and he remained improved for over three weeks. His pre-test history showed spontaneous remissions.)

Increasing the dosage of Dibenamine from 5 to 7.5 mg/kg of body weight was associated with an increase in the degree of immediate and 18 hour improvement.

Correlations were sought between initial neurologic status and initial abnormal autonomic functions, and between change in neurologic status and changes in autonomic functions. Because the series was small, statistically significant correlation is difficult. However, a significant coefficient of correlation was found between neurologic improvement and increase in hand temperature after Dibenamine. For the 12 infusions, $r = .5$, $t = 1.9$, and $p = .08$; and for 11 infusions (omitting Subject 4, whose response was atypical), $r = .74$, $t = 3.3$, and $p = .01$. Similar coefficients computed on the changes after sodium amytal are very low.

(G) *Psychiatric Examinations.* Without going into any details, the group of patients may be described as representative of the negative variety of schizophrenic reactions—catatonic type, moderately severe. There was no psychiatric change following saline, but sodium amytal brought about definite lessening of tensions. With the verbal mobilization that developed in 4 subjects after amytal, many schizophrenic (hebephrenic and para-

Suppression of Local Tissue Reactivity (Shwartzman Phenomenon) by Nitrogen Mustard, Benzol, and X-Ray Irradiation.

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When a filtrate of *Eberthella typhosa* is injected intradermally into a rabbit and 24 hours later injected intravenously, there develops a severe hemorrhagic necrotic reaction at the site of the intradermal injection within a period of 2-6 hours. Shwartzman¹ made his original observations on this reaction in 1927. It was soon found² that many unrelated bacteria were capable of producing the reaction, including the *Neisseria intracellularis* (Meningococcus), as well as non-bacterial substances, such as agar and starch. Although a certain small number of rabbits are naturally resistant to the reaction, a filtrate or endotoxin of a virulent meningococcus will consistently produce strong reactions in 90-100% of rabbits tested.

Although the histological reaction is basically one of vascular injury, opinions^{2,3} differ as to the nature of the Shwartzman phenomenon. There is agreement, however, that it is a non-specific reaction which does not involve the known immunological mechanisms (agglutinins, precipitins, etc.). Because of the known depressive effect of Benzol, X-ray irradiation, and nitrogen mustards on the parenchymatous elements of the blood-forming organs⁴⁻⁷ and on the reticuloendothelial

system,⁸⁻¹⁵ these agents were employed in an effort to throw light on the nature of the Shwartzman reaction.

Methods and Materials. The meningococcus filtrate and meningococcus endotoxin which were used throughout this study were prepared as follows:[†]

Meningococcus filtrate: Avirulent Type I strain of meningococcus was grown in 16 oz. flat-sided bottles for 24-30 hours on casein digest agar¹⁶ enriched with cysteine. The growth of each bottle was washed off with 10 cc of physiological saline in 0.4% phenol and centrifuged at approximately 3000 RPM for 20 minutes. The clear supernatant was passed twice through Berkefeld candle filters, adjusted to pH 7.8 and cultured for sterility.

Meningococcus endotoxin: The remaining sediment was resuspended in water (20 cc per culture bottle), adjusted to pH 8.2, incubated for 2 hours, placed in the icebox overnight, readjusted to pH 7.8, placed in a water bath at 60°C for 1 hour and 45 minutes, and cultured for sterility.

Fresh batches of the filtrate and the endotoxin were prepared approximately every 3

⁸ Hektoen, L., *J. Inf. Dis.*, 1915, **17**, 415.

⁹ Hektoen, L., *J. Inf. Dis.*, 1916, **19**, 69.

¹⁰ Simonds, J. P., and Jones, H. M., *J. Med. Res.*, 1915, **33**, 197.

¹¹ Hektoen, L., and Corper, H. J., *J. Inf. Dis.*, 1921, **28**, 279.

¹² Spurr, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 259.

¹³ Philips, F. S., Hopkins, F. H., and Freeman, M. L. H., *J. Immunol.*, 1947, **55**, 289.

¹⁴ Taliaferro, W. H., and Taliaferro, L. G., *J. Inf. Dis.*, Feb., 1948, **82**, 5.

¹⁵ Jaffe, R. H., *Physiol. Rev.*, 1931, **11**, 227.

[†] Kindly furnished by the laboratory of Dr. C. Phillip Miller.

¹⁶ Boor, A. K., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 22.

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¹ Shwartzman, G., *J. Exp. Med.*, 1927, **46**, 497.

² Shwartzman, G., *Phenomenon of Local Tissue Reactivity*, Paul B. Hoeber, Inc., 1937, p. 60.

³ Rich, A. R., *The Pathogenesis of Tuberculosis*, Chas. C. Thomas Co., pub., 1946, p. 425.

⁴ Seeling, L. E., and Osgood, E. E., *Downey's Handbook of Hematology*, Vol. IV, Paul B. Hoeber, Inc., pub., 1938.

⁵ Krumbhaar, E. B., and Krumbhaar, H. D., *J. Med. Res.*, 1919, **40**, 497.

⁶ Pappenheimer, A. M., and Vance, M., *J. Exp. Med.*, 1920, **31**, 71.

⁷ Kindred, J. A., *Arch. Pathol.*, 1947, **43**, 253.

drug at the same time were found to have priapism. The subjects had not been examined for this in the previous tests.

Comments: There is psychiatric dissatisfaction with schizophrenia as a nosologic entity and particularly with the sub-classifications as they now stand. The stigmata characteristic of catatonia are physiologic and can be experimentally produced or abolished. When sodium amytal or Dibenamine dissolves the catatonia in patients with schizophrenic reactions, catatonic type, hebephrenia or paranoia appears. Catatonia, which is an abnormal physiologic state, is therefore actually at a different descriptive level from either hebephrenia or paranoia, which are psychopathic states. Further discussion of this matter is reserved for later publication.

There is considerable experimental and clinico-pathological evidence suggesting that the manifestations of catatonia are mediated by the hypothalamus²⁵ and/or the basal ganglia.²⁶ We will not review this material in the present paper. However, it can be pointed out that both motor and autonomic centers are located in this region of basal arterial supply, and that motor and autonomic disturbances frequently accompany clinical and experimental lesions in this area. Catatonia and epidemic encephalitis supply many examples of co-existence of such motor and autonomic abnormalities. The physiologic basis for the association of these abnormalities may be the common blood supply to the hypothalamus and the basal ganglia.

Summary: 1. Certain autonomic functions (B.P., P.R., skin temperature, palmar

sweat response, electrical skin resistance), the signs of catatonia, and the psychiatric status of 9 patients with schizophrenic reactions, catatonic type, were evaluated before and after saline, sodium amytal, and Dibenamine infusions.

2. Pre-injection autonomic abnormalities were low systolic B.P., cool extremities, and either very low sweat responses with high E.S.Rs., or hyperhidrosis with very low E.S.Rs.

3. Saline produced no significant changes.

4. Sodium amytal infusions were followed by increased hand temperatures and improvement in the catatonia, especially in the verbal spheres. The subjects became relaxed, showed little interest in surroundings. In those 4 who developed sufficient speech function, hebephrenic or paranoid ideas were expressed. All changes were transient.

5. Dibenamine infusions were followed by a slight increase in systolic B.P. and a large increase in P.R., neither of which changes have been reported in non-psychotic subjects after Dibenamine. Excessive sweating was inhibited, and low E.S.Rs. were raised. The hands became warmer. Significant improvement in the signs of catatonia was noted in most subjects, especially (and in contrast to the effects of amytal) production of increased spontaneous activity and increased interest in surroundings. There was correlation between warming of the hands and the neurologic improvement. As with amytal, verbalization revealed hebephrenic and paranoid ideas. Toxic effects were infrequent and minor. All effects were transient, but of longer duration than with amytal.

6. The possible mechanisms by which Dibenamine inhibits sweating and by which it improves catatonia are discussed.

7. A difference in the descriptive level of catatonia from that of hebephrenia and paranoia is pointed out.

8. The common blood supply of the hypothalamus and the basal ganglia is suggested as a possible basis for the clinical association of certain motor and autonomic abnormalities.

²⁵ (a) Ingram, W. R., and Ranson, S. W., *Arch. Neurol. and Psychiat.*, 1934, **31**, 987. (b) Ingram, W. R., Barris, R. W., and Ranson, S. W., *Arch. Neurol. and Psychiat.*, 1936, **33**, 1175. (c) Ranson, S. W., and Ranson, M., *Arch. Neurol. and Psychiat.*, 1939, **42**, 1059.

²⁶ (a) Lewy, F. H., *Die Lehre vom Tonus u. Bewegung*, Berlin, Julius Springer, 1923. (b) Assoc. for Res. in Nerv. and Ment. Dis., *Diseases of the Basal Ganglia*, New York, The Williams and Wilkins Co., 1942. (c) Mettler, F. A., *J. Neuropath. and Exp. Neurol.*, 1945, **4**, 99.

TABLE I.
Effects of Benzol and X-ray Irradiation on the Shwartzman Phenomenon.

Dosage schedule			Reactions following inj. I.V. reacting factor (4-6 hr)			
Amt.	Days before I.V. react- ing factor*	No. of rabbits	None	++	++++	Deaths
Benzol—1.0-1.5 cc per kg daily	4	1				1
Subcut	7	4	2			2
	8	3	3			
	9	1	1			
	13	1	1			
2.0 cc per kg	7	1				1
	7	1	1			
Totals		12	8	0	0	4
X-ray total body irradiation						
800r	0	2			2	
300r	3	1			1	
800r	3	2		2		
800r	4	10	6	4		
800r	7	1		1		
Totals		16	6	7	3	

None = no reaction.

++ = suppressed hemorrhagic reaction (1 cm).

++++ = severe hemorrhagic reaction (3-6 cm).

* = Meningococcus filtrate or endotoxin.

TABLE II.
Effect of Single Injection of Methyl-bis Nitrogen Mustard Intravenously on the Shwartzman Phenomenon.

Dosage schedule			Reactions following inj. I.V. reacting factor (4-6 hr)			
Amt.	Days before I.V. react- ing factor*	No. of rabbits	None	++	++++	Deaths
2 mg	0	2			2	
kg	1	2			2	
	2	2	1	1		
	3	9	9			
	4	4	3			
	8	2	1	1		1-1 hr
	11	2		1		
	12	1			1	
	15	1			1	
1 mg	3	2	2			
kg						
0.5 mg	3	4			3	1-4 hr
kg						(no reac.)
Controls		21	1		19	1-4 hr
						(no reac.)

None = no reaction.

++ = suppressed hemorrhagic reaction (1 cm).

++++ = severe hemorrhagic reaction (3-6 cm).

* = Meningococcus filtrate or endotoxin.

suppression of the Shwartzman reaction was obtained in all 8 of the rabbits tested. Four rabbits, of the original 12 started with in this group, died during the period of benzol preparation.

(4) . Following Nitrogen Mustard. Nitro-

gen mustard (methyl bis chlorethyl amine hydrochloride) was given in a single injection by the intravenous route with doses ranging from 0.5-2.0 mg/kg body weight, from 0-15 days before injection of the intravenous reacting factor. As can be seen from Table II,

weeks and routine weekly cultures of both were sterile. The endotoxin was slightly more potent intravenously but both were found equally effective in eliciting severe hemorrhagic reactions in over 90% of the rabbits used. The control animals tested in any given group of rabbits received the same dose of the same substance by the same route as the treated animals of that group. The majority of the animals received the filtrate as the intradermal injection (*the skin preparatory factor*) and the endotoxin intravenously (*the intravenous reacting factor*).

Animals: Male albino rabbits weighing 2-3 kg were used. The hair on the abdomen was removed with electric clippers. No depilatories were used; no shaving was done.

Intradermal injections: Intradermal injections of 0.3 cc of the undiluted meningococcus filtrate or endotoxin were made in 3 different areas, the epigastrium, the right and left lower quadrants. The primary reactions resulting from the intradermal injections usually consisted of erythema and varying degrees of edema which would fade gradually over a 48-hour period if not followed in 24-48 hours by injection of the intravenous reacting factor.

Intravenous injections: From 20-26 hours after the intradermal injection, 2 cc of the undiluted meningococcus endotoxin per rabbit or 2 cc of the undiluted meningococcus filtrate per kg body weight was injected intravenously through the ear vein. A small percentage of the rabbits died within the first few hours after this injection from the inherent toxicity of the undiluted filtrate or endotoxin. Dilution was avoided to insure as high a percentage of severe reactions as possible.

Results: The effects of various agents were studied to determine their suppressive action on the Schwartzman phenomenon.

Results in Untreated Control Animals. Twenty-one normal untreated rabbits were used as controls. Of these, 19 developed severe hemorrhagic necrotic reactions measuring 3-6 cm in diameter at all 3 sites of the intradermal injections. One rabbit of the 21 was resistant and developed no reaction and

the other rabbit died at the end of 4 hours post intravenous and had no skin reaction at the time of death. There was no consistent correlation noted between the intensity of the primary reaction from the intradermal injection itself and the severity of the hemorrhagic reaction after the injection of the intravenous reacting factor.

Results in Treated Animals. (1) The following reagents employed in near-lethal doses exerted no suppressive action on the Schwartzman reaction: benadryl, (10 mg IV q 3 h), urethane, (1 Gm bid subcut, for 1-14 days), penicillin G, (500,000 u and 1,000,000 u IM daily x 4 days), crude penicillin, (35 cc bid x 2 days), streptomycin, (0.5 Gm and 1.0 Gm daily IM x 4 days), British anti-lewisite, (50-100 mg bid subcut), sodium ascorbate, (75 mg daily subcut x 10 days), alpha tocopherol, (25 mg subcut daily x 10 days), protamine, (20 mg bid IV x 2 days), toluidine blue, (20 mg bid IV x 2 days), and mapharsen (13 mg/kg IV 1 inj.). Total thyroidectomy 3 weeks before the reaction and bleeding (30 cc daily x 2 days) also had no suppressive effect.

(2) **Following Total Body X-ray Irradiation.** When 13 rabbits were exposed once to 800 r total body irradiation 3-7 days before injection of the intravenous reacting factor there was almost complete suppression of the reaction in 7 of the rabbits and complete suppression of the reaction in the other 6 (Table I). One rabbit received a total body dose of only 300 r, 3 days before, and showed no suppression of the reaction. Two rabbits received 800 r total body irradiation apiece immediately before the injection of the intravenous reacting factor and these likewise showed no suppression of the reaction.

(3) **Following Benzol.** Benzol mixed with equal parts of sterile olive oil, was injected subcutaneously in doses of 1.0-2.0 cc of benzol per kg body weight (2.0-4.0 cc per kg of the benzol-olive oil solution) once daily for 7-13 days before injection of the intravenous reacting factor. The benzol was given daily until the rabbits showed outward signs of toxicity such as weight loss, refusal of food and lethargy. As noted in Table I, complete

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Conglutinating Complement Absorption Test Compared with Hemolytic Complement-Fixation Reactions Using Q Fever Immune Bovine Serum.

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The difficulty of evaluating serodiagnostic tests performed on animal serums of various species is well known. In complement-fixation tests sensitivity is largely sacrificed for specificity, and modifications, such as alterations of the units of complement, varying the time and temperature of the incubation period, have not proved entirely satisfactory.

Hole and Coombs¹ reinvestigated the phenomenon of conglutination, first described by Bordet and Streng,² and developed a conglutinating complement absorption test³ which they applied to a study of glanders infection in horses.⁴ This test is based on the observation that when heat-inactivated bovine serum and fresh (unheated) horse, cat or pig serum are added to a suspension of sheep red cells, the cells clump together, or conglutinate. Bovine serum plays a dual role by furnishing a natural antibody to the sheep cells for their sensitization, and also a substance termed conglutinin. The reaction of the antibody and sheep cells has the property of absorbing complement. Obviously, the complement in this case must be of a nonhemolytic nature, otherwise the sheep cells would be lysed rather than conglutinated. Fresh horse, cat or pig serum provides such a complement. Thus, conglutinin acts on the sensitized cells which have also absorbed complement and causes them to conglutinate. It is necessary that all 4 of the above components be present before conglutination occurs. In the test proper a

known antigen and a suspected immune serum are mixed in the presence of a conglutinating complement. Later the conglutinating system (inactivated bovine serum and sheep red cells) is added to detect the presence of unabsorbed complement. In this test, conglutination may be considered analogous to hemolysis in the hemolytic complement-fixation test.

In the report which follows, the test was applied to Q fever infections in cattle and the results were compared with those obtained by two widely used technics of the hemolytic complement-fixation reaction.

Technics of the Tests. Conglutinating Complement Absorption Test (C.C.A.T.) Diluent. Physiological salt solution adjusted to pH 7.3 with 0.005 M phosphate buffer was used throughout the study.

Sheep red cell suspension. Defibrinated sheep's blood was stored in modified Alsever's solution.⁵ Before use the cell suspension was standardized spectrophotometrically and diluted to 0.25%.

Conglutinin. As mentioned previously, bovine serum served as the source both of conglutinin and antibody for sheep red cells. Routinely bovine serum is titrated for conglutinin only. Since the sheep cell antibody is always much higher in titer, a sufficient amount of antibody to sensitize the cells is present in the quantity of serum required to cause conglutination.

Conglutinin is not destroyed at ordinary inactivation temperatures. It is easily preserved by sealing small volumes in glass ampoules and, without adding chemical preservatives, storing in the CO₂ icebox. For titration, the serum is thawed, inactivated for 30

¹ Hole, N. H., and Coombs, R. R. A., *J. Hyg.*, 1947, 45, 480.

² Bordet, J., and Streng, O., *Zbl. Bakt., I. Orig.*, 1909, 49, 260.

³ Hole, N. H., and Coombs, R. R. A., *J. Hyg.*, 1947, 45, 490.

⁴ Hole, N. H., and Coombs, R. R. A., *J. Hyg.*, 1947, 45, 497.

⁵ Kent, J. F., Bukantz, S. C., and Rein, C. R., *J. Immunol.*, 1946, 53, 37.

there was no protection afforded when the mustard was given either at the same time as the intravenous reacting factor or when it was given on the day of the intradermal injection 20-26 hours before the intravenous reacting factor. When 2 mg/kg of nitrogen mustard was given 2 days before the intravenous reacting factor, there was partial suppression of the reaction in 1 rabbit and complete suppression in the other. When the nitrogen mustard was given in doses of 1-2 mg/kg 3-4 days before the injection of the intravenous reacting factor, the effects of the nitrogen mustard became more pronounced and there was complete suppression of the reaction in 14 of the 15 rabbits so treated, with the 15th rabbit dying during the first hour after injection of the intravenous reacting factor. When given 8-11 days before, the suppressive effect of the mustard was beginning to wane and in only one of 4 rabbits was there complete suppression, in 2 rabbits there was partial suppression and in 1 rabbit there was no suppression. When given the mustard 12 and 15 days before the injection of the intravenous reacting factor, the rabbits had completely regained their ability to react and, like the control rabbits, had severe hemorrhagic reactions. When a 0.5 mg/kg dose of mustard was used, no suppressive effect was noted.

Discussion. It is considered significant that the Shwartzman phenomenon of local tissue reactivity, even though a non-specific reaction, can be completely suppressed by nitrogen mustard, benzol, and X-ray, whose individual effects on blood-forming organs and the reticulo-endothelial system are so nearly identical. It is postulated that the mechanism of suppression by these agents is exerted through their specific but common suppressive action on the reticulo-endothelial system, primarily the vascular endothelium. These endothelial cells being rendered anergic are not able to react to the active principles in a way that otherwise would be self-destructive.

The Shwartzman phenomenon may then be interpreted as a local intracellular defensive but self-destructive reaction of the vascular endothelium to the bacterial active principles

and not a reaction resulting from the direct toxic effects of these bacterial products on the cells. Thus; when the ability of the cell to react is interfered with or suppressed in a specific way, as exerted by nitrogen mustard, benzol, or X-ray, the integrity of the cell is maintained and a destructive process avoided.

It is suggested that this study provides an experimental basis for a new therapeutic concept in the treatment of diseases involving tissue and vascular reactivity to known and unknown toxins. This concept would be directed toward suppressing the ability of the vascular endothelium to react adversely to whatever circulating toxin might be the inciting agent. A group of these diseases would include active rheumatic fever, acute, subacute, and chronic disseminated lupus erythematosus, periarteritis nodosa, generalized vascular diseases due to hypersensitivity reactions to drugs, sera or vaccines, and probably dermatomyositis, rheumatoid arthritis, and acute and subacute glomerulonephritis. A recent case report by Osborne and associates¹⁷ of the successful response of a case of chronic disseminated lupus erythematosus to nitrogen mustard would seem to provide confirmatory evidence in support of the therapeutic concept postulated above.

Summary. 1. The Shwartzman phenomenon was studied in rabbits using a meningococcus filtrate and meningococcus endotoxin. 2. The following agents were without suppressive effect on the Shwartzman phenomenon: benadryl, urethane, crude penicillin extract, penicillin G, streptomycin, mapharsen, BAL, vitamin C, alpha tocopherol, thyroidectomy, and partial exsanguination. 3. The reaction was completely suppressed by pretreatment of the rabbits with nitrogen mustard or benzol. It was also completely suppressed in some rabbits and partially in others by pretreatment with total body X-ray irradiation. 4. It is suggested that this study provides an experimental basis for a new therapeutic concept in the treatment of diseases involving tissue and vascular reactivity.

¹⁷ Osborne, E. D., Jordon, J. W., Hoak, F. C., and Pschierer, F. J., *J.A.M.A.*, Dec. 27, 1947, 135, 1123.

TABLE I.

Results of C.C.A.T. and Complement-fixation Titrations of Positive Q Fever Cattle Serums with Q Fever, Murine Typhus and Rickettsialpox Antigens.

No. of serums tested	C.C.A.T. titers*				Complement-fixation titers*			
					Warm-bath method		Ice-box method	
	Q fever	Murine typhus	Rickettsialpox	Control†	Q fever	Control	Q fever	Control
1	1:16	—	—	—	—	—	1:16	—
1	1:64	—	—	1:8	—	—	1:8	—
2	1:64	—	—	—	—	—	1:8	—
							1:16	
1	1:128	—	—	1:32	—	—	1:16	—
1	1:128	1:16	—	1:64	—	—	1:32	1:8
1	1:128	—	—	—	1:16	—	1:64	—
2	1:128	—	—	—	1:8	—	1:32	—
1	1:256	—	—	1:8	1:16	—	1:32	—
3	1:256	—	—	—	1:16	—	1:32	—
							1:64(2)	
1	1:512	1:8	—	1:128	—	—	1:64	—
2	1:512	—	—	—	1:4	—	1:64	—
					1:8			
1	1:512	1:16	—	1:32	1:8	—	1:128	—
1	1:512	—	—	—	1:8	—	1:128	—
4	1:512	—	—	—	1:16	—	1:64(3)	—
							1:128	
5	1:512	—	—	—	1:32	—	1:64(2)	—
							1:128(3)	
1	1:1024	—	—	—	1:16	—	1:256	—
2	1:1024	—	—	—	1:32	—	1:128	—
							1:256	
3	1:1024	—	—	—	1:64	—	1:128	—
							1:256(2)	
1	1:2048	—	—	1:16	1:32	—	1:256	—

* The highest dilution of the serum in which complete, or near-complete (3+ or 4+) fixation was observed is shown.

† The dose of complement used is not always the same for each antigen. Serum controls were always tested with the highest dilution of complement used. It is reasonable to assume that if a serum is not anticomplementary in the presence of a small amount of complement it would not be anticomplementary with larger quantities of complement.

Unbuffered physiological salt solution, and 2% suspension of red cells are used. Varying amounts of amboceptor are titrated in the presence of a 1:30 dilution of complement for one hour at 37°C. Complement is titrated in the presence of 2 units of antigen after fixation for the same period of time and temperature; 2 units of amboceptor and cells are added and a period of one hour at 37°C is allowed for hemolysis. Two "full units" of complement contained in 0.2 ml are used in the test.

The test consists of mixing twofold serial dilutions of the suspected immune serum with 2 units of antigen and 2 "full units" of complement. Fixation is allowed to take place for one hour at 37°C. The sensitized cells are then added and the tubes reincubated for one

hour at 37°C. All tubes are stored overnight at 4 to 6°C and readings are made the following morning.

Icebox method. This method is similar to that described above except that 2 "exact units" of complement are used and a period of 24 hours at 4 to 6°C is allowed for fixation. The tubes are read after the addition of the sensitized cells, followed by incubation of one hour at 37°C.

In both complement-fixation tests the antigens were titrated with their respective immune guinea pig serums.

On each serum specimen all 3 titrations, the C.C.A.T. and the 2 hemolytic complement-fixation tests, were performed on the same day with the identical dilutions. The C.C.A.T., using Q fever, murine typhus and rickettsial-

minutes at 56°C and diluted 1:30 with the diluent. Graduated amounts are placed in tubes, brought to total volumes of 1.2 ml with diluent, and complement (0.4 ml of a 1:40 dilution), and cells (0.4 ml) are then added. Following an incubation period of 30 minutes at 37°C, the tubes are centrifuged for a few minutes at a low speed. After shaking the tubes, the conglutinated cells appear as small clumps in a clear supernate. The absence of conglutination is evident from the homogeneous suspension of cells. The smallest quantity of conglutinin giving complete conglutination is taken as one unit. Four units, contained in 0.4 ml, are employed in the test.

Conglutinating complement. Fresh horse serum, preserved by storing small amounts in sealed glass ampoules in the CO₂ icebox, was used. Its activity was satisfactory even after a period of several weeks. For titration, increasing amounts of complement (in increments of 0.05 ml) of an appropriate dilution are added to 0.4 ml antigen, with sufficient diluent to bring the total volumes to 1.2 ml. After incubation for 30 minutes at room temperature, 0.8 ml of a mixture of equal parts 0.25% sheep cells and bovine serum (adjusted to contain 4 conglutinating units) is added to each tube. Following a 30 minute incubation period at 37°C the tubes are centrifuged, shaken and read. The smallest amount of complement giving complete conglutination is taken as one unit. Two units, contained in 0.4 ml, are employed in the tests.

Antigens. The Q fever antigen, consisting of washed, formalinized suspension of the American Nine Mile strain of *Coxiella burnetii*, was prepared according to the method described by Plotz⁶ but with certain modifications, the details of which have been presented elsewhere.⁷ The antigens of *Rickettsia typhi* (murine typhus) and *Rickettsia akari* (rickettsialpox) were similarly prepared and used to control specificity. These two were used because at the time they were the only washed rickettsial body antigens available.

The 3 antigens were originally titrated with their respective immune guinea pig serums. The Q fever antigen was also titrated with a positive bovine serum which gave titers comparable to those obtained with the guinea pig serum.

The "optimal" antigen dose must be used with the C.C.A.T. because of the tendency for prozone reactions. This dose was determined by cross titration of various dilutions of the antigen in the presence of various dilutions of the immune serum. The smallest amount of antigen showing maximal conglutination titers, without demonstrating prozones, is the optimal dose.

Serums. Immune serums from 34 Q fever infected cattle were obtained through the courtesy of Dr. R. J. Huebner of the National Institute of Health, Bethesda, Md.

Technic of the C.C.A.T. Twofold dilutions of the inactivated serum to be tested are prepared. Two units of complement and the optimal antigen dose are added and the tubes incubated at room temperature for 30 minutes. After the addition of the conglutinating system the tubes are incubated at 37°C for an additional 30 minutes. The tubes are then centrifuged and read. Appropriate controls for the test serum, antigen and complement are always included.

In the original technic of Hole and Coombs³ 0.4 ml of each test component was used, thus making a total volume of 2.0 ml per tube. In the present study one-half these volumes were used and no interference with the sensitivity, specificity or the reading of the results was observed. Also, preliminary trials indicated that the results were equally satisfactory when stored cells and buffered diluent were used, instead of the fresh cells and unbuffered physiological salt solution suggested in the original method.

Hemolytic Complement-Fixation Methods. Two methods were used, the so-called warm-bath method, suggested by Huebner,⁸ and the icebox method.

Warm-bath method. Test components: serum, antigen, complement, amboceptor and fresh sheep red cells in 0.2 ml quantities each.

⁸ Huebner, R. J., personal communication.

⁶ Plotz, H., *Science*, 1943, **97**, 20.

⁷ Wolfe, D. M., Kornfeld, L., and Cox, H. R. to be published.

to the C.C.A.T. show titers from 4 to 8 times higher than those obtained with the icebox complement-fixation method, and these in turn are from 4 to 8 times higher than those given by the warm-bath method. A similar picture is noted in Table II with human and guinea pig Q fever immune serums. Here, however, the differences in titers are of an order of from 2 to 4 times greater rather than 4 to 8. This apparently greater increase in sensitivity when testing cattle serum is, as yet, unexplained.

The question arises whether the use of cattle serum, as source of the immune body, causes irregularities in the results of the C.C.A.T. One would expect that conglutinin and normal antibodies for sheep cells introduced in excess of the standard amounts would tend to lower the antibody titer rather than cause the observed increase. Normal sheep amboceptor probably was present in the serums studied but no attempt was made to adjust the amount of amboceptor employed in the test. It is doubtful whether any conglutinin persisted until the date of testing since storage conditions are unfavorable for that particular component, and bearing in mind its relatively low titer (1:40 or 1:48), dilution of the serums beyond that point undoubtedly removed any effects which may be attributed to the introduction of additional conglutinin into the reaction mixture.

It is noteworthy that the titers of the Q fever, rickettsialpox and murine typhus antigens, as determined by the icebox complement-fixation method, were 1:32, 1:32 and

1:16 respectively, while the "optimal" C.C.A.T. titers on the same antigens were 1:120, 1:4 and 1:4 respectively. The Q fever antigen titer of 1:120 was determined with both bovine and guinea pig immune serums.

From the icebox complement-fixation results obtained with the Q fever negative-Brucella negative cattle serums (Table III) it would seem possible to adopt a lower limit of test significance, *i.e.*, a positive result exhibited in serum dilutions only as high as 1:8 would not be considered significant. But the inadvisability of imposing such an arbitrary limitation is demonstrated by the higher titers offered by the Q fever negative-Brucella positive serums (Table III).

The data presented indicate that the Q fever conglutinating complement absorption test may prove superior, both in sensitivity and in specificity, to either of the hemolytic complement-fixation tests employed to date. It is realized that numerous specimens of serum from cattle suffering from infections other than Q fever and Brucellosis should be examined before placing too much confidence in the results of the C.C.A.T. Such a study is now being undertaken and serums of goats, sheep, swine, dogs and horses will be included.

Conclusions. Preliminary studies in testing Q fever immune bovine serums indicate that the conglutinating complement absorption test is both more sensitive and more specific than are either the warm-bath or icebox methods for carrying out hemolytic complement-fixation tests.

TABLE II.

Results of C.C.A.T. and Complement-fixation Titrations of Positive Q Fever Human and Guinea Pig Serums with Q Fever, Murine Typhus and Rickettsialpox Antigens.

Serum	Lowest serum dilution tested	C.C.A.T. titers				Complement-fixation titers			
		Q fever	Murine typhus	Rickettsialpox	Control	Warm-bath method		Ice-box method	
						Q fever	Control	Q fever	Control
Human 1		1:1024	1:128	1:128	1:128	1:8	1:8	1:64	1:32
" 2		1:256*	—	—	1:8	1:64	—	1:128	—
" 3		1:512	—	—	—	1:64	—	1:128	—
" 4		1:512*	—	—	1:16	1:32	—	1:64	—
" 5	1:8	1:1024*	—	—	—	1:256	—	1:512	—
" 6		1:256	—	—	—	1:32	—	1:128	—
Guinea pig No. 3 Nine-Mile		1:2048	—	—	1:8	1:512	—	1:1024	—
Guinea pig No. 39 Nine-Mile		1:2048	—	—	—	1:512	—	1:1024	—
Guinea pig No. 5 Nine-Mile		1:2048	—	—	—	1:512	—	1:1024	—
Guinea pig Henzlerling		1:2048	—	—	1:32	1:512	—	1:1024	—

* Prozone.

TABLE III.

Results of C.C.A.T. and Complement-fixation Titrations of Serums from Cattle Having No Known Contact with Q Fever, with Murine Typhus, Q Fever and Rickettsialpox Antigens.

Serum	No. serums tested	C.C.A.T. titers				Complement-fixation titers			
		Q fever	Murine typhus	Rickettsialpox	Control	Warm-bath method		Ice-box method	
						Q fever	Control	Q fever	Control
Brucella negative	2	—	—	—	—	—	—	—	—
	6	—	—	—	—	—	—	1:4	—
	2	—	—	—	—	—	—	1:8	—
Brucella positive	3	—	—	—	—	—	—	1:8	—
	3	—	—	—	—	—	—	1:16	—
	1	—	—	—	—	—	—	1:32	—

pox antigens, and the 2 types of hemolytic complement-fixation tests, using Q fever antigen only, were performed on human, guinea pig and cattle serums. With the C.C.A.T. pools of normal human serums, human syphilitic serums and normal guinea pig serums (all used in dilutions of 1:4) were negative with the 3 antigens. Murine typhus and rickettsialpox guinea pig serums, tested with their respective antigens, gave titers comparable to those obtained with the icebox method for complement fixation test.

Discussion of Results. Considering the results of the Q fever agglutinating comple-

ment absorption test from the point of specificity, it is seen from the data shown in Tables I and II that, unless also anticomplementary, no Q fever immune serum (cattle or otherwise) reacted with the murine typhus or rickettsialpox antigens. Of the 17 serums obtained from cattle having a negative history of Q fever (Table III), none were positive when examined by the C.C.A.T.

It will be noted from the data summarized in Table I that with the positive Q fever cattle serums the order of increasing sensitivity is: the warm-bath method, the icebox method, and the C.C.A.T. Serums submitted

Serum Neutralizing Antibodies to the Infecting Strain of Virus in Poliomyelitis Patients.*†

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There has been very little satisfactory data collected regarding the development of specific neutralizing antibodies in human patients during an infection with one of the poliomyelitis viruses. Tests applied to convalescent or acute and convalescent sera, have usually been made with any convenient laboratory-adapted strain of virus. In most instances, workers have failed to demonstrate rising antibody titers, such as those encountered in many other virus infections, and frequently antibody has been found in high titer during the acute phase of the disease. These unusual and unexpected findings have been attributed usually to: (1) suspected changes in the virus strain, which might have occurred during adaptation to a laboratory animal, (2) the possibility that infection was due to a virus antigenically distinct from that used for the test, and (3) the possibility that the substance or substances responsible for neutralization are of a non-specific nature.

To our knowledge, only two instances have been reported in which a virus has been isolated from a patient and the same virus used to test the acute and convalescent phase sera of that patient for antibodies.^{1,2} In both instances the test was qualitative and served only to indicate roughly the presence or absence of antibody. The results were interpreted as indicating that in the *acute stage*, antibody was absent. Antibody was definitely present in the later specimen in the one instance,² but the results of the test on the

convalescent specimen were considered equivocal in the other.¹

Beginning 4 years ago, serum specimens and stool specimens were collected from a series of paralytic patients from various parts of California. A large series of viruses was isolated and monkey adaptation attempted, but unfortunately, most of the viruses had a low degree of pathogenicity for monkeys and could not be satisfactorily adapted to give clear-cut results in neutralization tests. At the present time, after using over 200 monkeys, tests of a reasonably satisfactory nature have been completed on the sera from 7 patients. Since the results are fairly consistent they are being reported at this time. Tests are now in progress on sera from other patients.

Methods. After isolation of a virus from feces, it was passed in monkeys as a 20% cord suspension, or 10-fold concentrate thereof, for not less than 3 passages, or until most of the inoculated monkeys developed paralysis. At this time, except in the earliest experiments, a pool of 20% cord suspension from several monkeys was concentrated in the ultracentrifuge to approximately one-tenth of its original volume and frozen in dry ice in a series of ampoules. Two or 3 monkeys were then inoculated with a mixture of an equal quantity of normal monkey serum and this frozen virus suspension. This mixture had been incubated one hour at 37°C, then overnight at 5°C; the predetermined conditions of the neutralization test to be performed later. If all monkeys became paralyzed, the virus was considered to be adequately adapted for the neutralization test. A number of viruses had to be discarded, after numerous tests showed that paralysis did not develop consistently.

Following the preliminary assay of pathogenicity, the virus was tested with each of 2

* Aided by a grant from the National Foundation for Infantile Paralysis.

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¹ Trask, J. D., and Paul, J. R., *J. Exp. Med.*, 1933, **58**, 531.

² Sabin, A. B., *J.A.M.A.*, (June 28), 1933, 749.

or 3 sera from the patient, together with other controls of normal monkey serum. The serum-virus mixtures were incubated as described above, and finally inoculated intracerebrally in monkeys using 2, 3, or 4 monkeys for each serum-virus mixture. The total volume inoculated was usually 1.0 cc, but in a few instances 1.5 cc was used. Development of clinically observable paralysis and of typical pathologic lesions was used as a criterion of failure to neutralize. Monkeys not observed to have paralysis were considered negative and were not sacrificed for sections. Such monkeys were considered to have been "protected". Acute phase sera from the first 3 patients appeared to neutralize the virus. The tests were repeated and the results confirmed. However, in doing so one serum from each series was either used completely, or was lost through an accident. Thus serial dilutions of sera could not be performed, although it became obvious at this time that with antibodies present at the onset, such a method must be employed, if possible differences in titers were to be demonstrated. In the last 4 cases, serial 5-fold dilutions of serum were tested against the concentrated virus suspensions. In order to save monkeys, the sera were usually tested first in a 1:3 or a 1:5 dilution, since undiluted sera in earlier tests had always protected. Normal monkey serum controls were made at the lowest serum dilution used for the human sera.

Results. In the first 3 cases, children with spinal paralytic types of infection, ages 3 to 9, the undiluted acute phase sera taken on the 2nd, 4th and 6th days after onset neutralized the virus as did the convalescent sera. Not less than 4 monkeys were employed for each acute phase serum. Table I presents the results of the last 4 cases, showing age of patient, type of paralysis, number of days after onset of illness when each serum was drawn, and the paralysis ratio of monkeys inoculated. The first serum was frequently drawn before, or at the time of onset of paralysis, usually on the same day as the stool specimen from which the virus was isolated. This generally corresponded to the first or second day of hospitalization.

TABLE I. Monkey Neutralization Tests with Acute and Convalescent Phase Sera from Patients, Tested Against Homologous Viruses Isolated from the Same Patients.

Patient and virus	Age of patient yr.	Type of paralysis	Results* monkey serum controls	Patient's sera					
				Acute		Convalescent			
				Days after onset	Serum dilution	Results*	Days after onset	Serum dilution	Results*
A.K.	6	Spinal	4/4	5	Undil.	0/2	74	Undil.	0/2
					1:5	0/2		1:5	0/2
					1:25	2/2		1:25	0/2
					1:125	—		1:125	2/2
G.P.	16	"	3/3	4	1:5	1/2	24	1:5	0/4
					1:25	2/3		1:25	3/4
					1:3	0/3	80	1:3	0/3
					1:15	3/3		1:15	0/3
Camp.	2	"	4/4	4	1:75	1/2		1:75	0/3
					1:3	0/3		1:3	0/3
					1:15	2/3	45	1:15	0/3
					1:75	3/3		1:75	0/3
Mo	15	bulbar	4/4	4					

* Numerator represents number of monkeys developing paralysis and the denominator the number inoculated.

Discussion and conclusions. In every instance the acute phase serum gave practically complete protection to all monkeys against a 20% monkey cord suspension which had usually been concentrated 10-fold by ultracentrifugation. A larger series of patients may show some without this titer of antibodies at, or before the onset of paralysis as did a group we reported who appeared to have been infected with a Lansing-like strain,³ but among this group of 7 patients, ranging from 2 to 16 years of age, antibodies were definitely present either before infection or had already developed by the time of onset of paralysis.

The method of using serial 5-fold dilutions against a constant amount of virus, employed in the last 4 cases, appears to be satisfactory for this type of study and continued tests of this nature should afford important immunologic data on poliomyelitis. We realize that it would have been desirable to have known the titer of virus used in each instance, but we were influenced by consideration of the number of monkeys involved. It is obvious, how-

ever, from the results with serial serum dilutions, that the titer of virus used was neither in excess nor insufficient for the information sought.

Among the 4 cases in which serum titrations were made there is an apparent increase in antibody titer by the 45th, 74th and 80th days, but no definite rise in titer is apparent in the one case tested on the 24th day. In one instance the demonstrated increase in titer was only 5-fold, but in the others the increase was 25-fold or greater. It becomes apparent, therefore, that in poliomyelitis, as in most other virus infections, an increase in neutralizing antibodies usually develops as a result of infection. This finding encourages one to believe that a *type specific* serological test for this disease would have practical application. The frequent presence of specific antibody at readily detectable levels at the onset of paralysis or at the time of hospitalization lends further evidence against the possible usefulness of serum therapy, and possibly even serum prophylaxis. Obviously the results of these tests on man carry far more weight than tests on monkeys and chimpanzees following artificially induced infections.

³ Hammon, W. McD., and Izumi, E. M., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 424.

16683

Hyaluronidase Inhibitors in Body Fluids in Normal and Disease States.

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(Introduced by W. J. Nungester.)

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Because of the probable relationship of hyaluronic acid to the formation and integrity of connective tissues, a relationship between hyaluronic acid metabolism and a large group of connective tissue diseases has been suspected, particularly in the case of rheumatic fever, rheumatoid arthritis, and disseminated

vascular disease. The technical problems involved and the conflicting evidence which has been forthcoming have been extensively reviewed by Meyer.¹

This report deals with attempts to relate this group of diseases to hyaluronidase activity, and, more significantly, with the apparent association of many malignant states in humans with disturbances of hyaluronidase inhibitors in the sera of such patients.

* The Rackham Arthritis Research Unit is supported by a grant from the Horace H. Rackham School of Graduate Studies of the University of Michigan.

¹ Meyer, K., *Physiol. Rev.*, 1947, **27**, 335.

Method. The method used depends on the observation of Seastone² that capsules of a group C streptococcus isolated from guinea pigs consist largely of hyaluronic acid and that hyaluronidases from a variety of sources exert a destructive effect on the capsule which is a function of time and enzyme concentration.

This principle has been utilized previously by McClean³ for *in vivo* studies. In a discussion of methods for hyaluronidase estimation Meyer¹ has stated that the decapsulation method is unsatisfactory because of spontaneous decapsulation of the test organism.

The technic as now modified has proved to be free of this objection. It offers a highly sensitive method for quantitative assay of hyaluronidase concentrations in the range between .001 and .0001 viscosity reducing unit per cc. Since turbidity or viscosity of the test materials do not interfere, the method is particularly adaptable to comparative studies of hyaluronidase inhibitors in serum and other body fluids in normal and disease states.

The organism is maintained by daily transfer on rabbit blood agar plates. Only well isolated, large (1 mm diameter) mucoid colonies are fished for subculture to agar or broth. After overnight incubation, these surface colonies may reach as much as 3 mm diameter, and are soft, mucoid, and hemolytic. Blood agar cultures over 24 hours old are not suitable for transplanting to serum broth.

A heavy inoculum, consisting of from 5 to 10 large well isolated, mucoid colonies, is transferred to a tube of Difco Brain Heart Infusion broth enriched with 10% sterile heated (56°C/20 minutes) hog serum. The culture is incubated at 37°C for 4 to 6 hours. After incubation the culture tubes are centrifuged at high speed for 5 to 10 minutes, and the sediment resuspended in 2 to 3 cc of M/15 phosphate buffer, pH 6.5. This is the indicator suspension of encapsulated organisms. It is to be emphasized that in a properly prepared suspension every streptococcal chain is heavily encapsulated.

As Seastone found, capsules are better pre-

served during drying and fixing in the presence of blood or serum. This step can be eliminated by coating slides with a thin coat of Mayer's egg albumen before dropping loops of bacterial suspension on them. The slides are then dried in air and stained with Wright's stain by the usual technic.

The enzyme used in these studies was a dried testicular hyaluronidase assaying 230 VRU/mg, supplied to us by Drs. VonderHeide and Rodney of the Parke-Davis Research Laboratories. A single batch has been used throughout these studies. However, other preparations from the Schering Laboratories gave comparable results.

To study enzyme inhibition 0.2 cc of enzyme containing 1.0 VRU/cc and 0.1 cc of the serum or its serial dilutions were added to oven-dried 13X90 mm tubes. Racks were shaken and then 0.1 cc of organism suspension was added to the tubes. Inhibition was expressed as the highest dilution of serum inhibiting the decapsulation of the test organism for 90 minutes.

Rheumatic Disease. Guerra⁴ has reported that salicylates are capable of inhibiting the spreading action of hyaluronidase in skin. Using concentrations of buffered sodium salicylate from 5 to 100 mg%, no inhibition of hyaluronidase was demonstrable. (Table I) Inasmuch as Meyer⁵ has described a gentisic acid conjugate of salicylate in urine capable of inhibiting hyaluronidase, the urine and serum of patients taking large doses of salicylates were tested for hyaluronidase inhibitors.

TABLE I.
Inhibition of Testicular Hyaluronidase *in vitro*.
Each tube: Enzyme 0.2 cc 1.0 VRU/cc
Organism susp. 0.1 cc
Drug 0.1 cc

	Conc. of drug mg %			
Sod. salicylate conc.	100	50	25	5
Inhibition obtained	0	0	0	0
Para amino benzoic acid conc.		50	25	5
Inhibition obtained		0	0	0
Gold sod. thiosulfate conc.	1.0	0.5	0.25	0.1
Inhibition obtained	0	0	0	0

⁴ Guerra, F., *Science*, 1946, 103, 686.

⁵ Meyer, K., and Ragan, C., *Fed. Proc.*, 1948, 7, 173.

² Seastone, C. V., *J. Exp. Med.*, 1939, 70, 361.

³ McClean, D., *J. Path. and Bact.*, 1942, 54, 284.

TABLE II.
Inhibition of Testicular Hyaluronidase by Body
Fluids Before and After Oral Sodium Salicylate.

	Dilution of body fluid			
	1:1	1:5	1:10	1:20
Normal serum	+	+	0	0
Normal serum after sod. sal.	+	+	0	0
Normal urine	0	0	0	0
Urine after sod. sal.	0	0	0	0

Time: 90 min.

Conc. of enzyme 1.0 VRU/cc.

+ = capsules present.

(Table II) In the case of urine no inhibitor capable of inhibiting 1.0 unit per cc of enzyme could be found, indicating that in therapeutically occurring concentrations salicylate conjugates presumably present in urine had no significant inhibitory effect on this reaction.

In serum, which normally contains hyaluronidase inhibitor, no increase could be produced by the ingestion of salicylates (Table II). These observations, therefore, confirm the observations of Pike⁶ and Dorfman⁷ and co-workers, who were unable to demonstrate hyaluronidase inhibition *in vitro* unless concentrations of salicylate of 2 g% were used.

Similarly, gold sodium thiosulfate and para aminobenzoic acid, drugs with an apparent influence on the course of certain connective tissue diseases, were without effect in producing enzyme inhibition when employed in therapeutically occurring blood serum concentrations. (Table I).

The presence of hyaluronidase inhibitor in normal serum has been described by several observers, but few quantitative observations have been made in disease states. Haas⁸ described an inhibitor in the sera of normal and diseased individuals. However, there were no distinct differences between rheumatic fever and several other diseases listed. The inhibitor substance was about equally active against like amounts of pneumococcus type VI hyaluronidase and bovine testicular extract.

Friou and Wenner⁹ were unable to find such inhibition of testicular hyaluronidase using the mucin clot prevention method, but found inhibition of streptococcal hyaluronidase in rheumatic fever, post-streptococcal convalescents as well as many normals. It is apparent from a perusal of this data that the inhibitor described was not the same as that which we have found, differing in the following characteristics: 1. The inhibitor described by Friou was not present in all sera, being absent from many normal sera. In our studies all sera have been inhibitory to some extent, although the variation among normals is marked. 2. The inhibitor was active against streptococcal hyaluronidase and inactive against hyaluronidase of testicular origin. In our studies the inhibitor was demonstrably active against testicular hyaluronidase as well as streptococcal enzyme. 3. The inhibitor was present in preserved sera as much as 10 years old and did not appear to decline in fresh sera on storage for 1 month. The inhibitor with which we deal is destroyed by heating to 56°C for 20 minutes and will disappear gradually in refrigerated sera within a few weeks. Inasmuch as the mucin clot prevention technic used by Friou requires relatively large amounts of substrate and enzyme, it is possible that the inhibitor present in all fresh sera toward testicular hyaluronidase was masked by its relative insignificance.

With these differences in mind, it is therefore proper to consider the testicular hyaluronidase-antihyaluronidase in a new relationship. As can be seen from the scatter diagram (Fig. 1) sera of various normal individuals of either sex vary widely in their ability to inhibit varying concentrations of testicular hyaluronidase. Since the same quantities of untreated serum, buffer, and bacterial substrate were present in each tube, these variations cannot be accounted for by variations in salt-concentration. It is clear that any conclusions relative to the type of hyaluronidase inhibitors discussed here, in normal as compared to disease states must

⁶ Pike, R. M., *Science*, 1947, 105, 391.

⁷ Dorfman, A., Reimers, E. J., and Ott, M. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 357.

⁸ Haas, E., *J. Biol. Chem.*, 1946, 63, 163.

⁹ Friou, G. J., and Wenner, H. A., *J. Infect. Dis.*, 1947, 80, 185.

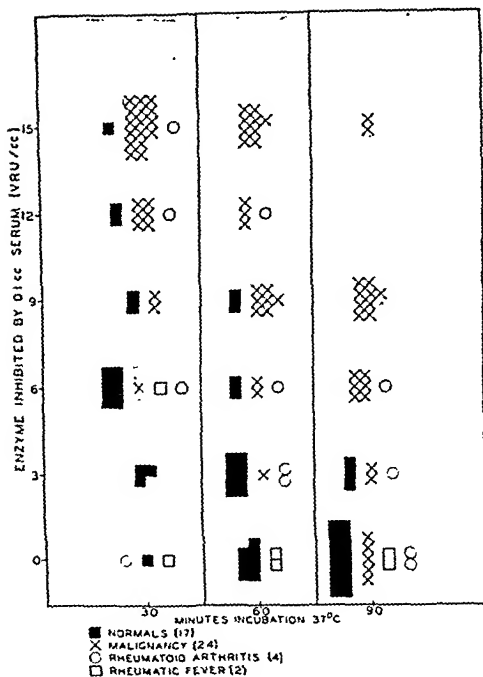


FIG. 1.
Relative testicular hyaluronidase inhibition by
normal and disease sera.

take into account the wide range of normal variation.

When the sera of 4 active rheumatoid arthritis sera and 2 active and convalescent rheumatic fever sera were tested during the same test run with the group of 17 normals, they fell within the normal range. Because of the known instability of the inhibitor in stored sera, no attempt could be made to compare the sera of an active rheumatic fever patient with the same patient's convalescent serum weeks later. It seems evident that if changes do occur during the convalescence they are not outside the normal variation, which in itself remains unexplained. We, therefore, do not find evidence that either rheumatic fever or rheumatoid arthritis are typified by a clear cut disturbance in the particular hyaluronidase inhibitor with which this reaction is concerned.

Malignancy. Hyaluronic acid has been isolated from malignant tumors by Kabat¹⁰

and by Meyer and Chaffee.¹¹ The presence of spreading factors (? hyaluronidases) in extracts of many animal and human tumors has been reported by Duran-Reynals.¹² The formation of spreading factor by malignant tissue has been regarded by Boyland and McClean¹³ as the cause of the greater permeability of such tissue to nutrients.

On the basis of the unpublished observation of Rodney¹⁴ that in Brown-Pearce carcinoma of rabbits a sharp rise in the titer of hyaluronidase inhibitor was detectable viscosimetrically in the sera of such animals, 24 sera of patients with a wide variety of malignancies both early and advanced and including leukemia and Hodgkin's disease were tested comparatively for hyaluronidase inhibitors with the same group of 17 control sera. As can be seen in the scatter diagram (Fig. 1), there is a statistical tendency for the cancer sera to show much greater inhibition under identical conditions. A few of the cancer sera show no characteristics which would distinguish them from normal sera, a few others are borderline, and some are markedly inhibitory. Since these observations have covered a relatively small group of normal, malignant, and other disease states, no conclusion can be drawn as to the exclusiveness of this property of excess inhibitor content for malignant sera, except to state that to date no other diseases studied have shown it.

Since testicular hyaluronidase was used throughout, to which no antibodies have been demonstrated, there is no evidence that an antigen-antibody reaction is involved, and the thermolabile nature of the inhibitor would suggest that it is not an antibody. The lack of differentiation between rheumatic states and normals would further suggest that the reaction is distinct from that described by Friou.

Conclusions. 1. A reliable biological method

¹¹ Meyer, K., and Chaffee, E., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 797.

¹² Duran-Reynals, F., and Stewart, F. W., *Am. J. Cancer*, 1931, **15**, 2790.

¹³ Boyland, F., and McClean, D., *J. Path. and Bact.*, 1935, **41**, 553.

¹⁴ Rodney, G., personal communication.

¹⁰ Kabat, E. A., *J. Biol. Chem.*, 1939, **130**, 143.

for the quantitative assay of small quantities of hyaluronidase or hyaluronidase inhibitor in the presence of body fluids is described.

2. Presumably, the conjugates of salicylates as well as salicylate itself in therapeutic concentrations have no inhibitory effect on testicular hyaluronidase *in vitro*.

3. Thermolabile hyaluronidase inhibitor has been found increased in a significant proportion of the sera of human malignancies in a small series studied.

4. Rheumatic states do not differ from the normal with respect to this inhibitor toward testicular hyaluronidase.

16684 P

Calcification of Hypertrophic Epiphyseal Cartilage *in vitro* Following Inactivation of Phosphatase and Other Enzymes.*

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Mineralization of hypertrophic epiphyseal cartilage *in vitro*, first demonstrated by Shipley,¹ requires concentration of calcium ions and phosphate ions in excess of a critical ion product, assumed to approximate the solubility product of a calcium phosphate salt. The concept of calcification solely as a precipitation phenomenon, however, fails to account for the specific localization of the mineral.

Robison² demonstrated phosphatase in hypertrophic epiphyseal cartilage and achieved calcification by addition of phosphoric ester in minute amounts to media of subcritical ion product. Robison³ regarded the local factor as possibly enzymatic since calcification *in vitro* had not been observed in tissues heated beyond 60°C, nor after complete inhibition of phosphatase by various chemical agents.

Gutman and co-workers⁴ demonstrated phosphorylative glycogenolysis in hypertrophic epiphyseal cartilage and have contended that calcification *in vitro* utilizes this enzyme system. On the other hand, McLean *et al.*⁵ have found little correlation of anaerobic glycolysis with calcification.

The present study was undertaken to determine whether or not enzymes are necessary for *in vitro* calcification.

Procedure. Slices of epiphyseal cartilage were taken from the long bones of young rats made rachitic on a modified diet 2965 of Steenbock and Black,⁶ and were subjected to varied preliminary treatment. The slices were then incubated approximately 18 hours at 37°C in isotonic media containing known amounts of calcium and phosphate at pH 7.4. Following incubation the tissues were washed in distilled water and stained with 2% silver nitrate. Experiments were designed to compare calcification in inorganic phosphate with calcification in phosphoric ester (sodium glycerophosphate). In addition, extracts of epiphyseal cartilage were prepared and were

* This work has been supported in part by research grants from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service; from the Josiah Macy, Jr. Foundation; and from the Williams-Waterman Fund of Research Corporation.

¹ Shipley, P. G., *Bull. Johns Hopkins Hosp.*, 1924, **35**, 304.

² Robison, R., *Biochem. J.*, 1923, **17**, 286.

³ Robison, R., and Rosenheim, A. H., *Biochem. J.*, 1934, **28**, 684.

⁴ Gutman, A. B., Warrick, F. B., and Gutman, E. B., *Science*, 1942, **95**, 261.

⁵ McLean, F. C., Lipton, M. A., Barron, E. S. G., and Bloom, W., unpublished data cited in *The Biological Action of the Vitamins*, University of Chicago Press; Chicago, 1942, p. 197.

⁶ Hess, A. F., Weinstock, M., Rivkin, H., and Gross, J., *Proc. Soc. Exp. Biol. and Med.*, 1929, **27**, 140.

analyzed for phosphatase activity by the method of Huggins and Talalay.⁷

Results. Incubation of slices in silver nitrate c. 1:1000 for one hour, followed by washing in distilled water for one hour, caused complete inhibition of calcification in phosphoric ester substrate. On the other hand, heavy calcification was observed when similarly treated slices were incubated in inorganic phosphate substrate. Untreated control slices calcified heavily in both media. Phosphatase activity of cartilage treated with silver nitrate was nil.

Similar results were observed when bichloride of mercury (c. 10^{-2} molar) was used as the inhibiting agent.

Incubation of slices in distilled water for one hour at constant temperatures ranging from 25° to 100°C (at 5° intervals) revealed that calcifiability in phosphoric ester substrate

was lost above 50° to 55°C as determined by subsequent incubation. Similarly, phosphatase activity decreased as the temperature increased, and was nil in tissues heated above 60°C. On the other hand, when inorganic phosphate substrate was used, calcification occurred in all tissues including those that had been boiled. In tissues heated above 65°C calcification was found to depend on a high concentration of medium: thus it was observed in media containing 1 mM calcium and 20 mM phosphate per liter, but it was absent when the phosphate concentration was less than 10 mM per liter.

Conclusions. 1. Calcification of hypertrophic epiphyseal cartilage *in vitro* is intrinsically non-enzymatic. 2. The phosphorylase and phosphatase systems in hypertrophic epiphyseal cartilage may have supplementary roles in the calcification mechanism, but they are not essential to the localized deposition of bone mineral.

⁷ Huggins, C., and Talalay, P., *J. Biol. Chem.*, 1945, 159, 399.

16685 P

Aureomycin in Treatment of Pneumococcal Pneumonia and Meningococcemia.*

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Aureomycin is a new antibiotic which is active *in vitro* and in experimental animals against gram-positive and gram-negative bacteria and is also effective against experimental infections with rickettsias and with viruses of the psittacosis lymphogranuloma venereum group.[‡] The findings in 4 cases of pneumococcal pneumonia and in one case of meningococcemia are presented here briefly. The bacteriologic methods are described elsewhere.^{1,2}

The cases of lobar pneumonia were of moderate severity and a single lobe was in-

volved in each instance. All were in males ranging in age from 16 to 65 years. Pneumococci were identified and typed from the

[‡] Data on isolation, pharmacology and activity *in vitro* and in experimental infections have been presented by workers of the Lederle Laboratories Division of the American Cyanamid Company at a Conference on Aureomycin, New York Academy of Sciences, July 21, 1948. Additional clinical and laboratory observations by the authors and by other workers were also presented at that conference.

¹ Paine, T. F., Jr., Collins, H. S., and Finland, M., *J. Bact.*, 1948, 56, 489.

² Collins, H. S., Paine, T. F., Jr., Wells, E. B., and Finland, M., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 174.

* Aided by a grant from the United States Public Health Service.

[†] Research Fellow, American College of Physicians.

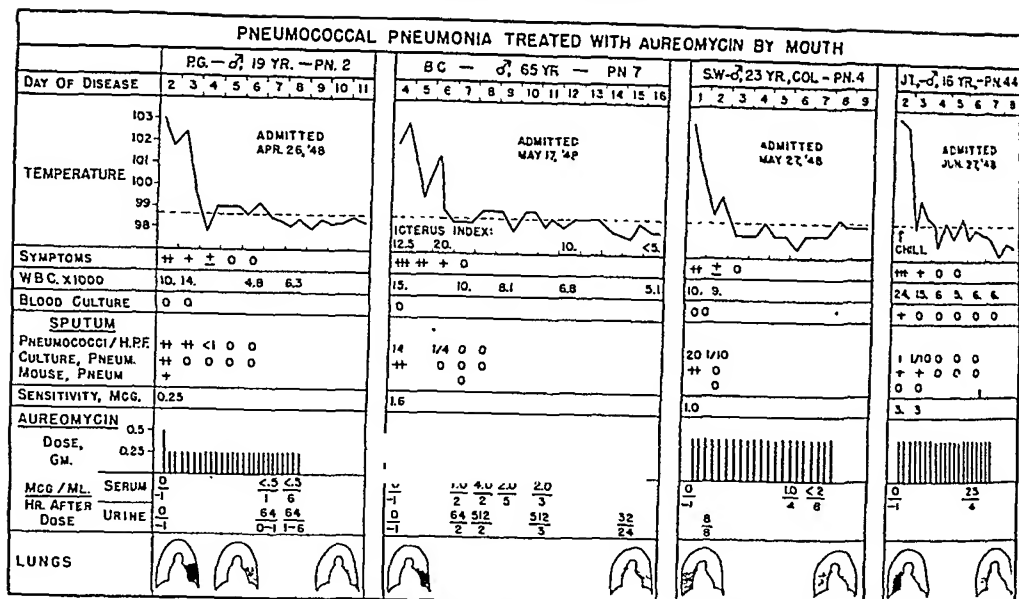


Fig. 1.
Relevant findings in 4 cases of pneumonia.

sputum in each instance and from the blood in one case before oral aureomycin therapy was started. One patient received an initial dose of 0.5 g followed by 0.25 g every 6 hours for a total of 5 g; the others received 0.5 g every 6 or 8 hours for a total of 10 g in 2 cases and 20 g in the oldest patient. No other chemotherapy or antibiotic was used. Some of the relevant findings are shown in Figure 1.

The patients became afebrile, and both subjective and objective improvement occurred in each instance between 18 and 36 hours after the first dose and was slowest in the oldest patients who had the severest illness and jaundice. The number of pneumococci decreased rapidly in the sputum following treatment and none could be demonstrated by any of the available methods, including mouse inoculation, after the second day of treatment. The pneumococci were completely inhibited by aureomycin in concentrations of 0.25 to 3.0 μ g per ml.

The relevant findings in the case of meningococcemia are shown in Figure 2. In this patient a tentative diagnosis of Rocky Mountain Spotted Fever was made on the basis of a history of tick bites, high fever, headache, slightly stiff neck and a rapidly spreading

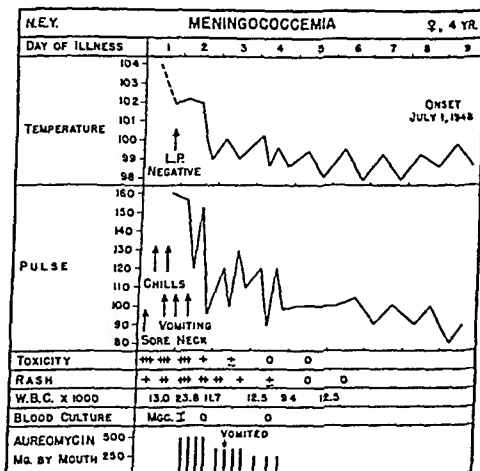


Fig. 2.
Relevant findings in a case of meningococcemia treated with aureomycin.

maculopapular eruption involving the trunk and extremities. Lumbar puncture yielded normal spinal fluid. Treatment was begun 12 hours after the onset of symptoms. The patient received 4 doses of 500 mg followed by 4 doses of 350 mg and then 3 of 250 mg, at 6-hour intervals. The patient was afebrile and much improved symptomatically and the rash had almost cleared within 18 hours after

first dose. A blood culture obtained before treatment was started yielded meningococcus, group I and subsequent blood cultures were negative.

The results in these cases appear to be comparable to those obtained in similar cases treated with penicillin or with effective sulfonamides. There were no toxic effects attributable to aureomycin in any of these cases.

Further experience is needed to determine the optimum dosage and duration of treatment as well as the effect in more severe cases. The present findings, however, suggest that aureomycin may be an effective agent in pneumococcal and meningococcal infections and warrants further clinical trial in these and other types of infections.

16686

Excretion of Androgens and 17-Ketosteroids in the Cebus Monkey Following the Administration of Testosterone Propionate.

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(Introduced by H. Selye.)

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An increase in urinary 17-ketosteroids and androgens after administration of testosterone has been noted in man,¹ chimpanzee,² Rhesus monkey,³ guinea pig⁴ and rat.⁵ Normally Cebus monkeys excrete minimal amounts of androgens as compared with other primates,⁶ hence we wished to examine their 17-ketosteroids and androgen excretion after administration of testosterone propionate.*

Material and Methods. Six adult male Cebus monkeys, weighing 2.8 - 3.3 kg, were used. After a control period of 6 days, each animal received daily 50 mg of testosterone propionate subcutaneously, in 1 ml of peanut oil solution, for 12 days followed by 5 days during which no treatment was given.

¹ Dorfman, R. I., and Hamilton, J. B., *J. Clin. Invest.*, 1939, **18**, 67.

² Fish, W. R., and Dorfman, R. I., *Endocrinology*, 1944, **35**, 22.

³ Dorfman, R. I., and Hamilton, J. B., *Endocrinology*, 1939, **25**, 28.

⁴ Dorfman, R. I., and Fish, W. R., *J. Biol. Chem.*, 1940, **135**, 349.

⁵ Dorfman, R. I., *J. Biol. Chem.*, 1938, **123**, xxx.

⁶ Valle, J. R., Henriques, S. B., and Henriques, O. B., *Endocrinology*, 1947, **41**, 335.

* Testosterone propionate was furnished through the courtesy of the Ciba Pharmaceutical Company of S. Paulo.

Throughout the period of observation (23 days) the urine of each monkey was continuously collected under toluene. Twice daily the urine specimen of all monkeys were pooled, acidified with 1% hydrochloric acid, placed in the refrigerator and extracted the following day. Hydrolysis, extraction and fractionation by Girard's reagent for chemical and biological assays was done as previously described.⁷ The urinary extracts from the control period of 6 days (I), the treatment period of 12 days (II) and the post-treatment period of 5 days (III) were kept separate for chemical and biological assays. Colorimetric estimations were done by the Zimmermann method as modified by Callow *et al.*⁸ and by the antimon trichloride technic.⁹ Assays of androgenic activity were made using the chick comb method.⁶

Results. A. Androgenic activity. In a preliminary test we verified that the androgenic activity of the urine collected during periods I, II, and III was approximately the same. Therefore the final assay was done only on

⁷ Henriques, O. B., and Henriques, S. B., *Mem. Inst. Butantan*, 1946, **19**, 11.

⁸ Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, 1938, **32**, 1312.

⁹ Pincus, G., *Endocrinology*, 1943, **32**, 176.

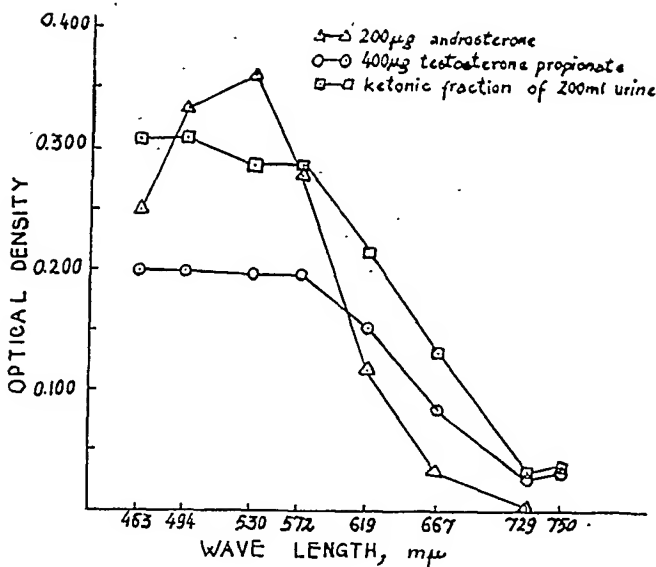


FIG. 1.
Comparison of absorption spectra of androsterone, testosterone propionate and ketonic fraction of urinary extract submitted to Zimmermann's reaction.

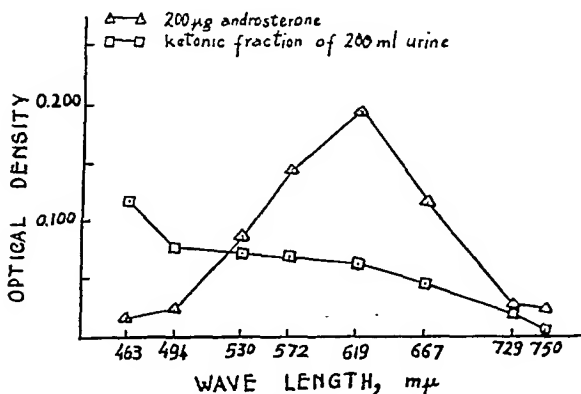


FIG. 2.
Comparison of absorption spectra of androsterone and ketonic fraction of urinary extract submitted to antimony trichloride reaction.

samples I and II. Androgen excretion was found to be 94.2 ± 24.5 μg per liter for sample I and 87.9 ± 39.0 for sample II. This corresponds to 10 and 6.5 μg per 24 hours, respectively, for one monkey.

B. 17-Ketosteroids. Using Zimmermann's procedure, the color obtained was brownish instead of violet. Therefore we analysed this color with the Pulfrich photometer. Fig. 1 shows the absorption spectra of androsterone, testosterone propionate and the ketonic frac-

tion from the extract of 200 ml of urine of untreated monkeys. Fig. 2 shows the absorption spectra of the same substances with the exception of testosterone, using the antimony trichloride reaction. It will be noted that there is no maximum absorption at 530 mμ for the urinary extract with the Zimmermann's reaction nor at 620 mμ with antimony trichloride procedure. After administration of testosterone propionate, no significant qualitative or quantitative change was detected in

the absorption spectra.

Discussion and Summary. As previously stated, the administration of testosterone to many animals including man and the Rhesus monkey, results in an increased urinary excretion of androgens and 17-ketosteroids. In contradistinction to this we noted no increase in either of these substances in the urine of treated Cebus monkeys. This finding parallels those of Dingemans and Tyslowitz,¹⁰ Paschkis *et al.*,¹¹ who found no increase in

¹⁰ Dingemans, E., and Tyslowitz, R., *Endocrinology*, 1941, **28**, 450.

the urine of dogs similarly treated. In dogs¹¹ and man¹² increased urinary estrogenic activity has been shown after testosterone treatment. Our results would suggest that in the Cebus monkey as in man and the dog, testosterone can be converted to estrogens, but further work is necessary to elucidate this point.

¹¹ Paschkis, K. E., Cantarow, A., Rakoff, A. E., Hausen, L. P., and Walking, A. A., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 213.

¹² Dorfman, R. I., and Hamilton, J. B., *Endocrinology*, 1939, **25**, 33.

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Determination of Creatine, Creatinine, and Related Compounds in Urine by Means of Paper Chromatography.*

STANLEY R. AMES AND HUGH A. RISLEY. (Introduced by Philip L. Harris.)

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A partition chromatographic procedure for the identification and estimation of creatine and creatinine and their demethylated analogues, glycoyamidine and glycoyamine (guanidoacetic acid) was devised during the course of an investigation of aminoaciduria in dystrophic humans.¹ Two different color-reactions were investigated to indicate the location of the various substances on the developed chromatogram. One was based on the Jaffe reaction, the formation of a reddish-orange product when alkaline picrate is added to solutions of creatinine and related compounds. This reaction has been used by Dent^{2,3} to identify creatinine on a paper strip following partition chromatography. The other was a modification of the Voges-Proskauer reaction in which diacetyl in alkaline solution forms a pink product with creatine and similar com-

pounds.

Method Using Jaffe Reaction. The Jaffe reaction is the conventional method for the detection of creatinine. It is dependent on a cyclic lactam structure such as is found in creatinine and glycoyamidine and is not given by the hydrated straight chain analogues, creatine and glycoyamine. It is necessary, therefore, to dehydrate the latter compounds *in situ* before applying the color reaction. It was found that heating the dry paper strips for one hour at 100-110°C transformed creatine to creatinine. Glycoyamine was dehydrated to glycoyamidine by heating for one hour at 100-110°C but the conversion was not quantitative. Further heating did not appear to increase the amount of glycoyamidine formed as evidenced by the depth of the color reaction. Therefore, in actual practice a constant heating period at a constant temperature was carefully followed in order to make valid comparisons between the depth of color formed with standard solutions and with urines of unknown composition.

Details of the experimental procedure are as follows: A 0.025 ml aliquot of urine or

* Communication No. 144.

¹ Ames, S. R., and Risley, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 131.

² Dent, C. E., *Biochem. J.*, 1947, **41**, 240.

³ Dent, C. E., in Reifstein, E. C., Conference on Metabolic Aspects of Convalescence, Josiah Macy, Jr. Foundation, New York, 1946, p. 126.

standard solution is placed dropwise at a starting point on a filter-paper strip. The strips are then developed for 15-20 hours using water-saturated solutions of butanol, phenol or lutidine-collidine (50-50) as solvents. The strips are air-dried, heated to 100°C for 1 hour and cooled to room temperature. The Jaffe reaction is obtained on spraying the strips with an alkaline picrate solution prepared by adding 10.0 ml of 10% NaOH to 50.0 ml saturated picric acid. The presence of creatinine or related compounds is evidenced by the appearance of an orange spot against a light yellow background. The color appears immediately and is comparatively stable. The strips are roughly evaluated in a manner similar to that suggested for amino acids.¹ The color intensity is estimated from "1" to "5" where "1" represents the faintest band observable and "5" represents an intense color. The d_w value for each band is calculated by multiplying its width in cm by the color intensity. The procedure is quantitized by modification of any one of a number of published procedures: Extraction of the material and analysis by micro-Kjeldahl,⁴ extraction of the colored product followed by conventional colorimetric analysis,^{2,5} or less precisely by spot dilution technics⁴ or by spot area technics.⁶

In order to test the accuracy and sensitivity of this procedure, the various compounds under consideration were analyzed individually, as well as in mixtures, and when added to urines. In Table I the average R_F values obtained in the various solvents are given. Water-saturated solutions of butanol and lutidine-collidine give a better separation of creatine and creatinine than a water-saturated phenol solution. However, phenol gives an excellent resolution between glycocyamine and the others. Differentiation of creatinine and glycocyamidine by this method is not feasible because of the close correspondence of their R_F values. The smallest amount of

TABLE I.
Average R_F Values for Creatine and Related Compounds.

Substance	Solvent (H ₂ O-saturated)		
	Butanol	Phenol	Lutidine-collidine
Creatine	.08	.85	.24
Creatinine	.33	.91	.56
Glycocyamine	.07	.66	.28
Glycocyamidine	.30	.89	.52

Creatine and creatinine were Eastman Kodak Co. products. Appreciation is expressed to Dr. F. Smith of the University of Rochester Medical School for a generous sample of glycocyamine. Glycocyamidine in all cases was obtained *in situ* by dehydration of glycocyamine by heat. The solvents were used as water-saturated solutions at about 23°C and the chromatograms were developed in a thermostated room at the same temperature. The R_F values are the averages of a large number of determinations using pure solutions, mixtures and samples of urine, and are reproducible to $\pm 2 R_F$ units.

creatinine or creatine (converted to creatinine by heating) which could be detected was 11 μ g. One hundred micrograms of glycocyamine heated to convert it to glycocyamidine yielded a barely distinguishable spot.

The method for the determination of creatine and creatinine is therefore quite sensitive and precise. It is also easy to perform and it is possible to determine creatinine and related compounds in pure solutions or in urines with equal facility. Urines from a number of species, including rats, rabbits and humans, have been analyzed successfully. In studying urines it is possible to test for both amino acids and creatinine and related compounds on the same strip or sheet by spraying the Jaffe reagent onto the paper which has previously been treated with ninhydrin. The sensitivity and resolution of the creatinine determination is not impaired in the above procedure.

Following the completion of this investigation, a paper appeared by Maw⁷ concerned with the determination of creatine by partition chromatography. In this procedure developed strips, after being airdried and heated in an

⁴ Polson, A., Mosley, V. M., and Wyckoff, R. W. G., *Science*, 1947, **105**, 603.

⁵ Naftalin, J., *Nature*, 1948, **161**, 763.

⁶ Fisher, R. B., Parsons, D. S., and Morrison, G. A., *Nature*, 1948, **161**, 764.

⁷ Maw, G. A., *Nature*, 1947, **160**, 261.

oven at 100°C, were sprayed with 0.5 N sulfuric acid and heated at 100°C for 1 hour. This was stated to result in the dehydration of creatine to creatinine, the creatinine then being tested for by the Jaffe reaction. The method did not prove to be satisfactory in our laboratory, since the paper strips charred and were unusable. We have since learned⁸ that Dr. Maw has been unable to satisfactorily reproduce this technic and is now following a procedure somewhat similar to ours with the exception that the strips are heated for 3 hours rather than 1 hour.

Method Using Diacetyl Reaction. The use of alkaline diacetyl for the determination of creatine proved to be applicable to paper chromatography. This method is specific for creatine and gives no color with creatinine. Therefore, the strips were not heated, but with this exception the procedure as previously outlined was followed. A 50-50 water-alcohol solution saturated with sodium carbonate was prepared by adding 15 ml of absolute alcohol to 20 ml of saturated sodium carbonate solution and removing the resulting precipitate by filtration. Two ml of diacetyl were added to 10 ml of the resulting filtrate. When this solution was sprayed onto the strips, the presence of creatine was indicated by pink spots against a yellow background. Too high a concentration of sodium carbonate caused the strip to turn brown obliterating the pink color of the reaction. Insufficient sodium carbonate reduced the sensitivity of the method. After drying at room temperature the strips were placed in the oven at 100°C

for exactly 5 minutes to hasten the color development which at room temperature is very slow. The smallest detectable amount of creatine was 33 µg, a sensitivity of about 1/3 that of the Jaffe reaction. Neither glycocyamine nor glycocyamidine in quantities up to 200 µg developed a color with the diacetyl reagent. The diacetyl reaction is given by several amino acids, and confusing results can be obtained on pathological urines. Its principal value is in the positive identification of creatine since it clearly differentiates it from creatinine.

Summary. A procedure for the identification and determination of creatine, creatinine, glycocyamidine and glycocyamine is presented. Following resolution by paper chromatography, the developed chromatograms are heated to 100°C for one hour to dehydrate creatine and glycocyamine *in situ* to creatinine and glycocyamidine respectively. The latter compounds and others of similar structure give an orange color on treatment with the Jaffe reagent (alkaline picrate).

The Voges-Proskauer reaction (alkaline diacetyl) for creatine was also investigated and found suitable but less precise.

R_F values for creatine, creatinine, glycocyamine, and glycocyamidine are given for water-saturated solutions of butanol, phenol, and lutidine-collidine (50-50).

The chromatographic method possesses the advantage that creatine and creatinine can be determined independently and directly rather than by a differential procedure as in the conventional methods.

⁸ Maw, G. A., personal communication.

Biological Studies with Arsenic⁷⁶.I. Preparation of Arsenic⁷⁶ by Pile Irradiation of Cacodylic Acid.

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(Introduced by L. O. Jacobson.)

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Preliminary investigations into the production of radioactive arsenic, for distribution studies in animals and for clinical trial, indicated the need for inorganic radioactive arsenical compounds of a higher specific activity than could be obtained by pile irradiation of As_2O_3 . This led to the consideration of the "Szilard Chalmers reaction."¹ Basically, this is a general reaction in which the recoil energy, imparted to the As nucleus upon neutron capture, ruptures the chemical bonds which hold it in the complex molecule, leaving it free and radioactive. D'Agostino² was able to separate arsenic⁷⁶ from cacodylic acid after bombardment by a radium-beryllium source, and Szilard³ stated that pile irradiation of cacodylic acid would give good yields of radioactive arsenic. Preliminary experiments confirmed this.⁴

Methods. Cacodylic acid $(\text{CH}_3)_2\text{AsOOH}$, is a colorless solid that is easily soluble in water. It melts at 200°C and is extremely stable, being unaffected by fuming nitric acid, aqua regia, or potassium permanganate.⁵

An accurately weighed quantity (300-750 mg) of this substance, finely powdered, is introduced into a soft glass vial (8 x 40 mm) that has a small glass eyelet fused to its rounded bottom. The open end of the vial is then sealed with paraffin end, eyelet end up, placed within a larger quartz tube (12 x

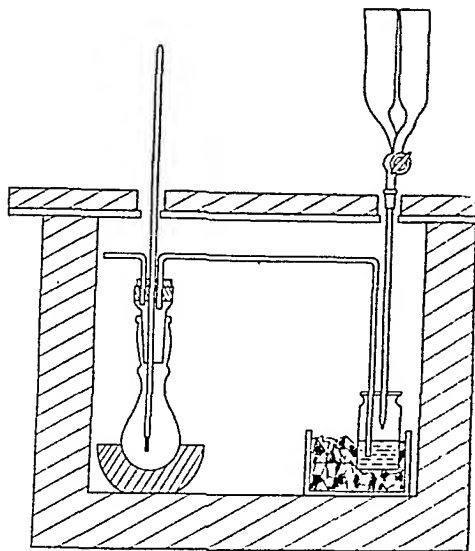


FIG. 1.
Cross section of lead shield with distilling apparatus in place.

125 mm). The latter is evacuated, sealed, and irradiated in a neutron pile.

Following irradiation, the quartz tube and its container are handled only by remote control apparatus and behind 10-15 cm of solid lead shielding. All chemical operations are carried on behind a U-shaped shield, having a floor and roof constructed of dovetailed, 10-cm thick, lead bricks, laid in alternating courses. A mirror, set at an angle at the open back of the shield, allows the operator to view the interior and the progress of the experiment. A cross section of the shield and the distilling apparatus it contains are shown in Fig. 1.

The irradiated quartz tube is slipped into a lead block placed behind the shield. The neck of the tube is then shattered by the remote control ratchet mechanism illustrated

¹ Szilard, L., and Chalmers, P., *Nature*, 1934, 132, 462.

² D'Agostino, O., *Gazzetta Chimica Italiana*, 1935, 65, 1075.

³ Szilard, L., personal communication.

⁴ Brues, A. M., ed., Quarterly Report, Biology Division, Argonne National Laboratory, August 1, 1947, ANL-4078.

⁵ Raiziss, G. W., and Gavron, J. L., *Organic Arsenical Compounds*, Chemical Catalog Co., Inc., New York, 1927.

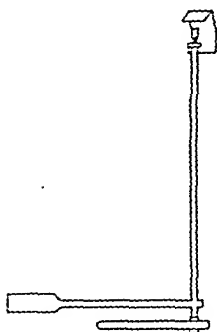


FIG. 2.

Remote control ratchet mechanism used in crushing the quartz capsule.

in Fig. 2. The "hot" cacodylic acid is transferred to the distilling flask with the aid of a right-angle hook that engages the eyelet on the bottom of the soft glass vial in which the acid is contained. The distilling vessel is an ordinary 100-ml Kjeldahl flask, the long neck of which is replaced by a 24-40 standard ground glass taper. The still head is the male portion of the taper, flared at the upper end to hold a No. 6, three-holed rubber stopper. A bent glass tube inserted through one hole admits compressed air, another holds the thermometer, and the third admits the end of the U-shaped distilling arm that leads to the receiving bottle.

For the usual sample of 500-750 mg of cacodylic acid, the distilling flask contains 7.5 cc of concentrated HCl (arsenic-free), and 0.4 g of cuprous chloride (CuCl). The cuprous chloride reduces any arsenic present in the pentavalent state to the trivalent form. Once the vial of cacodylic acid is added, the still head is sealed by manipulating the thermometer stem from above, through the roof of the shield. The flask is heated by a Glasscol* heating mantle and distillation is carried out to 110°C. Between 80-110°C the free trivalent arsenic is distilled as AsCl₃. The current of air, introduced through the still head, increases the yield. The distillate is caught in a graduated 30-ml bottle, containing 10 cc of cold, distilled water in a small ice-bath. The volatile AsCl₃ fumes, bubbling

through the liquid, are hydrolyzed to arsenious acid. Neutralization to the phenolphthalein end point (pH 8.3) with 6 N NaOH follows. The NaOH is introduced through the long-stemmed burette that pierces the shield roof.

Aliquots for activity measurements and arsenic determinations are obtained with pipettes inserted in the remote control pipetor.⁶ A 25-lambda micropipette withdraws a sample that is diluted in a 100-ml volumetric flask with 6 N arsenic-free nitric acid. Five cc of this solution is further diluted to the mark with nitric acid in another 100-cc volumetric flask. Four-cc samples of this final dilution are placed in duplicate Coors porcelain capsules and evaporated to dryness. These samples are then counted under a thin-window mica Geiger-Mueller tube and aluminum foil absorber of 54 mg/cm², using a conventional scaling and timer unit, and compared with a standard uranium source which is similarly mounted. Because of the high energy levels of the beta and gamma radiations of the arsenic sample (β max = 3.04, γ max = 2.15), mass absorption can be neglected.

The activity of the sample is calculated as follows:

$$\mu\text{c As}^{76}/\text{ml} = \frac{\text{c/s sample}}{\text{c/s standard}} \times \frac{1}{\text{Absorption factor Al foil} \times \text{dilution factor} \times 3.7 \times 10^{-4} \times \text{geometry factor of standard}}$$

The total amount of arsenic present, both stable and radioactive, is determined by means of the modified Gutzeit method for arsenic, as described in Scott's Standard Methods of Chemical Analysis.⁷ Usually 0.1 cc of the solution obtained is sufficient to give a 20-30 μg stain. Knowing this value and the activity of the sample, the "specific activity", *i.e.*, the ratio of the number of radioactive arsenic atoms to the total number of arsenic atoms, is obtained.

Results. Table I is a summary of several

⁶ Tompkins, P. C., Broido, A., and Teresi, J. D., *The Handling of Radioactive Materials in the Experimental Biology Section, Manhattan District Declassified Document (MDDC) 377.*

⁷ Scott, W. W., *Standard Methods of Chemical Analysis*, Van Nostrand Co., New York, 5th Edition, Vol. I, pp. 101-108, 1939.

* Obtainable from the Glasscol Apparatus Co., 1700 S. 7th Street, Terre Haute, Indiana.

TABLE I.
Yields of Radioactive Arsenic⁷⁶ from Pile Irradiated Cacodylic Acid (Szilard-Chalmers Reaction).

Amt. cacodylic acid irradiated (mg)	NaCl %	Activity/cc (mc)	Arsenic/cc (mg)	Total vol. (cc)	Total activity (mc)	Specific activity (mc/mg)
300	10	2.37	0.09	17.5	41.5	26.4
700	14	4.26	0.26	28.0	119.0	15.2
500	13.0	4.35	0.35	24	104.4	12.0
600	7.7	4.50	0.35	20	90.0	12.8
700	10.0	8.50	0.34	17	142.0	25.0
750	16.0	5.60	0.32	20	112.0	17.5
800	20.0	4.57	0.26	16	73.2	17.5
500	16.0	2.77	0.17	17	47.1	16.4
800	14.0	4.0	0.26	25	100.0	16.9
650	14.0	6.48	0.20	25	162	32.4
700	11	6.82	0.18	8	54.6	30.3

TABLE II.
Comparison of Specific Activities of Several Arsenic Compounds Exposed to Pile Irradiation.

Material	mc As ⁷⁶	Enrichment
	mg As ⁷⁵	
As ₂ O ₃	0.5	1
Cacodylic acid (CH ₃) ₂ AsO(OH)	15.0	30
Arsenilic acid		
C ₆ H ₄ NH ₂ AsO ₃ H	0.1	0.2

Values are representative ones.

preparations of arsenic⁷⁶ from pile irradiated cacodylic acid. There is wide variation in the specific activities because (1) time and power levels of pile irradiation were not uniform, and (2) activities were calculated to time of preparation of the arsenic solution.

It will be seen, however, that specific activi-

ties of arsenic⁷⁶ obtained by pile irradiation of cacodylic acid are considerably higher than those obtained by pile irradiation of arsenic trioxide. Table II compares specific activities following irradiation of cacodylic acid, arsenic trioxide, and arsenilic acid. In addition to the high specific activity of the resulting product, as can be seen from Table I, irradiation of cacodylic acid yields a solution of high total activity, this being a function of the high neutron capture cross section for As⁷⁵.

Summary. A method of obtaining As⁷⁶ of high specific activity by pile irradiation of cacodylic acid is described.

The assistance of Dr. Willard McCorkle, Mr. John Rose, and Dr. Alexander Langsdorf is gratefully acknowledged.

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Relation of Folic Acid and Vitamin A to Incidence of Hydrocephalus in Infant Rats.*

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Richardson and Hogan¹ observed hydro-

* Contribution from the Department of Agricultural Chemistry, Missouri Agricultural Experiment Station, Journal Series No. 1130.

This investigation was supported in part by a grant from the U. S. Public Health Service.

cephalus among infant rats and ascribed it to the inadequacy of the maternal diet. The diet, which was of the usual synthetic type, was supplemented with all of the recognized

¹ Richardson, L. R., and Hogan, A. G., *J. Nutrition*, 1946, **32**, 459.

vitamins except pteroylglutamic acid, and the abnormality occurred in about 2% of the offspring. When the diet was supplemented with aqueous liver extracts, or with an eluate prepared from liver extracts, the abnormality was not observed. These concentrates would be expected to contain folic acid, but it was observed that crystalline "*L. casei* factor" would not cure the defect once it had developed. More recently Richardson and De Mottier² have produced hydrocephalus in an unrelated colony of rats.

The purpose of this communication is to show the relationship of pteroylglutamic acid and vitamin A to the incidence of hydrocephalus among the offspring of rats that consume a synthetic diet.

Methods. Adult albino rats from the colony described by Richardson *et al.*¹ were placed on the experimental diets only a short time before mating since it was found that depletion periods of several weeks had no noticeable effect on the incidence of hydrocephalus. After pregnancy occurred the rats were transferred to individual round cages, containing wood shavings as litter, where the offspring were born. All of the offspring were allowed to remain with the dam until weaning. The young rats were observed frequently for abnormalities and were weighed at weekly intervals until the end of the fourth week, when they were weaned and discarded if no abnormalities occurred.

At the end of the second, third, and fourth weeks the rats were examined for symptoms of hydrocephalus by means of a light-transmission test. The head of the rat was placed over a small opening in a box containing an incandescent bulb, with the size of the orifice so adjusted that the head of a normal rat of comparable size transmitted no light. If transmission of light could be detected the animal was considered hydrocephalic, the severity being measured by the amount of light transmitted.

The basal synthetic diet was composed of acid-washed casein, 30%; cerelose, 52%;

wood pulp, 3%; salts,¹ 5%; lard, 10%; vitamin A, 2000 I.U.; vitamin D, 285 I.U.: (Vitamins A and D were supplied in the form of oleum percomorphum, Mead Johnson & Co.) α -tocopherol,[†] 2.5 mg; 2-methyl-1, 4-naphthoquinone, 2.5 mg; thiamine hydrochloride, 1.6 mg; riboflavin, 1.6 mg; pyridoxine hydrochloride, 1.6 mg; and calcium pantothenate, 4.0 mg. This diet was supplemented with various combinations of the following nutrients: Niacin, 5 mg; biotin, 0.020 mg; choline chloride, 100 mg; inositol, 100 mg; and ascorbic acid, 10 mg. Since none of these vitamins had any effect on the incidence of hydrocephalus, all of the rations containing any of these vitamins were grouped together and designated as Diet A. Diet A was supplemented with mcg of pteroylglutamic acid,[‡] 5% liver extract or 5% brewers' yeast. The vitamin A content of the diet was varied from 2000 I.U. to 100 I.U. per 100 g of diet.

Results. The data in Table I indicate that the addition of pteroylglutamic acid to Diet A markedly decreases the occurrence of hydrocephalus among the offspring.

During 1947 there were 1001 young born to dams maintained on Diet A and 10 or 1.9% of those weaned showed symptoms of hydrocephalus. Of the 1890 young from dams which received 50 mcg of pteroylglutamic acid per 100 g of diet only 2 developed the abnormality. During this same period 3 out of 1826 young rats born in the stock colony, which received a natural feedstuffs diet and served as a control, became hydrocephalic.

When it was observed that pteroylglutamic acid decreased the occurrence of hydrocephalus it was considered worthwhile to present all of our earlier data¹ as they have some bearing on the relation of pteroylglutamic acid to the

[†] The α -tocopherol, thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, choline chloride, niacin, biotin, inositol, ascorbic acid, and 2-methyl-1, 4-naphthoquinone were supplied through the courtesy of Dr. D. F. Green, Merek and Co., Rahway, N. J.

[‡] The pteroylglutamic acid was supplied through the courtesy of Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y.

² Richardson, L. R., and DeMottier, Jeanne, *Science*, 1947, 100, 644.

HYDROCEPHALUS IN INFANT RATS

TABLE I.
Relation of Pteroylglutamic Acid in the Maternal Diet to Hydrocephalus in the Offspring.

Experimental period	Supplements to diet ^A	No. of litters	No. born	No. weaned	Hydrocephalus	
					No. cases	%*
1947	None	157	1001	532	10	1.9
	Pteroylglutamic acid (50 meg)	218	1890	1437	2	0.1
	Stock ration†	230	1826	1212	3	0.2
1944-1946	None	895	5026‡	2986	52	1.7
	Liver extr.§	180	1197‡	937	0	0.0
	Brewers' yeast	83	488‡	309	3	1.0

* % of those weaned.

† A diet of natural feedstuffs, used as a control.

‡ The number of young retained, since in the earlier experiments the litters were reduced to 8.

§ The liver extract supplement consisted of either 5% of an aqueous extract of pork liver or 1% of a fullers' earth eluate of this extract.

TABLE II.
Relation of Vitamin A in the Maternal Diet to Hydrocephalus in the Offspring.

Vitamin A content*	No. of litters	No. born	No. weaned	Hydrocephalus	
				No. cases	%†
I.U. per 100 g					
2000	51	335	226	5	2.2
1000	33	220	122	3	2.5
500	38	217	145	3	2.0
100	40	244	145	1	0.7

* The vitamin E content of the diet at each vitamin A level was varied from 0.5 mg to 2.5 mg but since the differences were of no consequence the data are combined.

† % of those weaned.

abnormality. From 1944, when the first case was observed, through 1946 there were 5026 young retained from dams which consumed diets that contained no added folic acid. Fifty-two animals, or 1.7% of those weaned, developed the syndrome while of the 1197 retained from dams which received liver extracts rich in folic acid, none showed symptoms. There were 488 offspring retained from dams which received 5% dried brewers' yeast in the diet and 1% of those weaned developed hydrocephalus. The folic acid content of the brewers' yeast is not known but it must have supplied some of the vitamin. It seems probable that the quantity was insufficient to prevent the syndrome.

The occurrence of hydrocephalus among young rabbits has been observed by Hyde³ and by Greene,⁴ and it was believed by Greene to be the result of a vitamin A deficiency. In view of the possibility that destruction of vita-

min A might occur during storage of the rations, lower levels of vitamin A were fed in an attempt to increase the incidence of hydrocephalus. The results are shown in Table II.

The incidence of hydrocephalus was not increased by lowering the vitamin A content of the diet from the usual level of 2000 I. U. to as low as 100 I.U. per 100 g.

Although the incidence of hydrocephalus

TABLE III.
Moisture Contents of Rat Brains.

Type Diet	Brains Analyzed No.	Moisture Content %
Stock	23	79.93
Synthetic	23	79.91
"	6*	88.10

* Brains from hydrocephalic rats.

³ Hyde, R. R., *Am. J. Hyg.*, 1940, 31, 1.

⁴ Greene, H. S. N., Department of Pathology, Yale University, personal communication.

TABLE IV.
Cerebrospinal Fluid Pressure Determined by Cisternal Puncture.

Type Diet	No. of Observations	Cerebrospinal fluid pressure mm of water	Stand. dev.
Stock	30	77	26
Diet A	39	67	29
" A	3*	220	—
" A + PGA	34	78	29

* Rats showing symptoms of hydrocephalus.

on Diet A was 10 times as high as on the stock ration, it was still too low to permit rapid progress in discovering the cause. It was hoped that other less obvious symptoms of the deficiency might occur. Therefore a search was made for chemical and physiological differences between rats reared on synthetic and on practical rations. The moisture content of the brains from hydrocephalic rats is usually about 90%. That there was not a significant difference in moisture contents of brains of normal appearing rats from the synthetic and from the stock rations is shown by Table III. Twenty-three rats, 4 to 5 weeks of age, from each of the 2 types of rations were paired according to age, weight and sex, and their brains removed and dried for 24 hours at 65°C in a vacuum oven.

Hydrocephalus may be classified as internal or external depending on whether the fluid accumulates in the ventricles or in the subdural space and as communicating if there is free flow of liquid between the subdural space and the ventricles. In the communicating type there is a marked increase in cerebro-

spinal fluid pressure as determined by cisternal puncture.⁵ Using the technic described by Griffith and Farris the cerebrospinal fluid pressure of the hydrocephalic rats was found to range from 150 to 350 mm of water. Thus the hydrocephalus appears to be the communicating type. Cerebrospinal fluid pressure determinations were made on rats, 4 to 6 weeks of age and weighing from 60 to 150 g, reared on the stock diet and on synthetic diets with and without added pteroylglutamic acid. The results are shown in Table IV. The variation within a group was quite large and there was no significant difference between groups.

Summary. About 2% of the young rats weaned from dams fed a synthetic type diet deficient in folic acid developed hydrocephalus. This abnormality could be largely prevented by the addition of pteroylglutamic acid to the maternal diet. Decreasing the vitamin A content of the diet to a low level did not increase the incidence of the hydrocephalus. There was no difference in either the moisture content of the brains or in the cerebrospinal fluid pressure of rats from the stock colony and those from dams receiving a synthetic type ration.

⁵ Griffith, J. O., and Farris, E. J., J. B. Lippincott Co., 1942, pp. 192-196.

Antiproteolytic Activity of Human Serum with Particular Reference to Its Changes in the Presence and Considerations of Its Use for Detection of Malignant Neoplasia.*

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(Introduced by S. C. Harvey.)

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Lacking a specific test for malignant neoplastic disease (cancer), it is possible that something short of this with a high degree of correlation, analogous to the serological test for syphilis, will be of value in selecting patients for more detailed examination than that feasible in the customary screening process of a "Cancer Detection Clinic." Many investigators have reported tests with this object in mind, without producing convincing evidence of a sufficient correlation with the presence or absence of malignant tumor. A review of these contributions will be undertaken in a later detailed presentation. Many of these tests are concerned with alterations in coagulation of the blood or its capacity to inhibit various enzymes. Another similarity is that they tend to give false positive reactions in the same types of pathologic conditions, other than malignant tumors.

These observations led us to believe that the basic mechanism might be an alteration in the proteolytic-antiproteolytic balance of the serum. Brieger and Trebing¹ suggested the use of the antifibrinolytic reaction of serum as a test for malignancy. Other reports by Von Bergmann and Meyer,² Herzfeld,³

Roche⁴ and many others were critically reviewed by Weil.⁵

This approach was discarded because of the false positive reactions and the subjective nature of the test and the fact that it was considered a cachexia reaction. Contributions to our understanding of the antiproteolytic response since that time have been reviewed by Grob⁶ and Clark *et al.*⁷

A simple, reproducible, objective test of antiproteolytic (antitryptic) activity in serum was developed in this laboratory largely as a result of work by one of us (D.G.C.C.). This test is performed as follows.

Materials. 1. Veronal buffer: pH 7.4; Veronal 8.244 g; N/10 HCl 238 ml; Potassium oxalate .2948 g; NaCl 11.0 g; H₂O to 2000 ml.

2. Trypsin: A lyophilized pancreatic extract of bovine origin.

3. Fibrinogen: A lyophilized material of bovine origin almost free from lysin.

4. Thrombin: A material prepared according to the method of J. H. Milstone,⁸ from Armour beef plasma globulin ppt. pH 5.1 shipped in dry ice by air express. To 16 g of the Armour ppt. add 80 ml of veronal buffer. Grind well, as quickly as possible. Next add 0.1 N. NaOH with care to avoid an excess, to pH 7.4 (phenol red) and with alacrity in order to have as much as possible dissolved before the solution clots spontaneously. Centrifuge promptly at 2000 R.P.M. for 15 minutes,

⁴ Roche, M., *Arch. Int. Med.*, 1909, **3**, 249.

⁵ Weil, R., *Am. J. Med. Sc.*, 1910, **130**, 714.

⁶ Grob, D., *J. Gen. Phys.*, 1943, **26**, 405.

⁷ Clark, D. G. C., *et al.*, to be published.

⁸ Obtained from Armour and Company, Chicago, Ill.

⁹ We are indebted to Dr. Milstone for this method and for many other helpful suggestions.

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[†] National Research Council Fellow in Medical Sciences.

[‡] Fellow, American Cancer Society, as recommended by the Committee on Growth of the National Research Council.

[§] Jane Coffin Childs Fellow in Medical Research.
¹ Brieger, L., and Trebing, *Berl. klin. Woch.*, 1908, **45**, pt. 2, 1041, 1349, 2260.

² Von Bergmann and Meyer, *Berl. klin. Woch.*, 1908, **45**, 1673.

³ Herzfeld, E., *Berl. klin. Woch.*, 1908, **45**, 2182.

TABLE I.
Example Test Report.

Dilution	1:50	1:60	1:70	1:80	1:90	1:100	1:110	1:120	1:130	1:140	1:150
Test sera:											
A	+++	+++	+++	+++	0	0	0	0	0	0	0
B	+++	+++	+++	+++	+++	++	0	0	0	0	0
C	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

remove the supernatant and check to assure its pH of 7.4. Measure the supernatant and add 0.1 volume of .0275 M CaCl_2 , stirring constantly until coagulation is complete. Discard the fibrin and store the solution in the refrigerator for use within one week.

5. Serum: Obtained from the patient's blood with special care that all glassware and needles used are clean and dry.

Method. The test should be run within 6 hours after the blood is drawn. The serum is prepared in dilutions of 1:50, 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150. Solutions of trypsin, 35 mg to 100 ml of veronal buffer and fibrinogen 0.2 g to 40 ml of veronal buffer are prepared daily. The trypsin solution must be kept in the refrigerator. The thrombin is diluted 1:3 with veronal buffer and checked for activity as follows: to 0.1 cc of thrombin solution, 0.1 cc of fibrinogen and 0.3 cc of veronal buffer are added. A clot should form in 8 seconds. 0.25 cc of trypsin solution is added to 0.25 cc of serum dilution plus 0.3 cc of veronal buffer in 13 x 100 mm pyrex tubes. Allow to react 10 minutes at 25°C. Add 0.1 cc of fibrinogen, and allow to react 15 minutes at 36.5°C. Add 0.1 cc thrombin solution, and allow to react 15 minutes at 36.5°C. A constant temperature water bath is necessary. At this time the tubes are examined. The tube with the greatest dilution containing a definite clot is considered the end point. The end point is rarely in doubt, and in that case the test should be repeated in the doubtful range.

Using a large series of tubes it is necessary to add the reagents and read the results at specified intervals, usually 10 seconds, but trained personnel can cut the interval to 5 seconds with the desired accuracy. Results of repeated tests on the same serum within 6 hours or of samples from a normal individual

on successive days will not vary by more than one dilution.

For the present each series of tests is run with a normal control serum, and the report is made with both the actual dilution titre and the ratio of the unknown titre to the normal serum titre. The test run shown in Table I may be used as an example. Serum A, the normal, is recorded as 80 or 100%, serum B would then be 100 or 125% and serum C >150 or >187%. The percentages so obtained have been used in the discussion of results.

Discussion. In view of the accuracy and objectivity of this method, it was considered desirable to explore again the possibility of its having significance for the detection of malignant new growths. Consequently, the antiproteolytic titre of the serum was determined on all new patients admitted to the "Tumor Clinic" as well as upon many in the hospital with known or questionable malignant tumors and with various diseases, not neoplastic. Blood was accepted from all sources without accompanying definitive information as to the patient's disease.

The data obtained were assembled and correlated and a method of statistical analysis** applicable to this problem was developed and applied. This analysis revealed that titres below 112% of the normal control could be considered as negative for malignant tumors with a possible error of 5%, and that titres above 136% of the normal control could be considered as indicative of malignant neoplasia with a possible error of 5% whereas

** This was made by Mrs. Sylvia Johnson of the statistical division of the Tumor Registry with the assistance of Mr. David Votaw, Jr., of the Department of Mathematics of Yale University, to whom we are greatly indebted. The method employed will be reported elsewhere.

TABLE II.
Diagnosis.

% of normal titer	Malignant neoplasia	Benign neoplasia	No apparent disease	Disease other than malignancy	Malignant neoplasm after definitive therapy	
					No recurrence	Recurrence
75 negative	1	5	5	7	2	0
87 "	4	8	6	4	1	0
100 "	3	45	68	48	42	0
113 doubtful	10	15	15	22	14	1
125	10	4	1	7	5	0
137 positive	7	0	1	1	0	0
150 "	13	0	0	0	0	2
165 "	15	0	0	0	1	1
175 "	14	0	0	0	1	3
187 "	15	0	1	1	0	2
200 "	16	0	0	3	0	0
over 200	7	0	0	4	0	2
Total cases in each category	115	77	97	97	66	11

All cases studied are listed according to the category of disease and the ratio of their test titer to the normal titer.

TABLE III.

	Positive, %	Doubtful, %.	Negative, %
Malignant neoplasm	75	18	7
Benign neoplasm	0	25	75
Patients without apparent disease	2	17	81
Patients with diseases other than neoplasia	9	31	60
Post-treatment patients:			
1. Without recurrence	3	29	68
2. With recurrence	90	10	0

The percentages of patients in each group giving reactions in the positive, doubtful, and negative ranges are recorded.

with titres in the range between 112% and 136% of the normal control, the significance of the findings is doubtful. All patients with a definitive diagnosis on whom one or more tests had been made were reviewed. It is realized, of course, that complete certainty as regards the diagnosis will depend upon prolonged follow-up of these patients. 463 patients are included in this study, of whom 115 had malignant neoplasms, 77 had benign neoplasms, 97 were without apparent disease, 97 had demonstrable disease other than malignant or benign tumor and 77 had had malignant neoplasm for which they had received definitive therapy. From a study of Table II it is apparent that (a) no case of "benign neoplasia" gave a positive reaction, while 75% were negative and 25% were doubtful; (b) only two cases without apparent disease gave

a positive reaction while 81% were negative and 17% were doubtful; (c) of the patients with diseases other than neoplasia 9% gave positive, 60% negative and 31% doubtful reactions; (d) on the other hand, of the patients with known malignant neoplasia, 75% gave positive reactions and only 7% were negative, while 18% were doubtful. This material is summarized in Table III.

The impression has been gained that after presumed complete removal of the malignant tumor or definitive therapy by x-ray the titre slowly falls to normal and does not rise again unless there be a recurrence. Of the patients without apparent clinical recurrence only 3% gave a positive reaction, as compared with 90% of those with clinical recurrence.

No detailed account of the false negative or

false positive reactions can be given in this brief report. In general the false negatives fall into 3 classes: (1) patients with tumors of borderline malignancy; (2) patients with very small carcinomas; and (3) those with extensive terminal malignant disease, or rapidly spreading metastases. The false positives fall almost entirely into 3 groups: (1) patients with advanced tuberculosis; (2) patients who have been operated upon very recently; and (3) patients with active infections. Of the last, those in whom the streptococcus is the agent can be ascertained by the use of the streptococcal antifibrinolysin test (Christensen,⁸ and Boisvert⁹). Reactions in the doubtful range occur frequently with

serum from patients with other diseases (such as diabetes and lues) which gave false positive reactions with previous antifibrinolysin tests.

A non-specific antiproteolytic reaction has been outlined, the results of which when carefully analyzed give a high correlation between a positive test reaction and the presence of malignancy in the patient whose serum is tested. Certain false negative and positive reactions occur and careful study will be necessary to define these. It may be hoped that methods to exclude the effect of other specific antifibrinolytic factors, such as the antistreptokinase, will be developed with the ultimate goal of a specific reaction for malignancy. For the present, the reaction is of value as a screening mechanism with a degree of correlation warranting extremely careful observation in all patients with a consistently elevated titre.

⁸ Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

⁹ Boisvert, P. L., *J. Clin. Invest.*, 1940, **19**, 65.

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Streptomycin-Induced Chlorophyll-less Races of *Euglena*.

LUIGI PROVASOLI,* S. H. HUTNER,* AND ALBERT SCHATZ.†
(Introduced by Paul A. Zahl.)

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In the course of attempts to obtain with the aid of antibiotics pure cultures of algal flagellates and related colorless forms, it was noticed that after exposure to streptomycin (STM), normal green pure cultures of several strains of *Euglena gracilis*, used for orientation experiments, became colorless and remained so on serial transfer in light on STM-free media. The theoretical implications of this observation prompted further study.

A study was made of this bleaching as a function of (1) concentration of STM, (2) duration of exposure to STM, (3) exposure under proliferating and non-proliferating con-

ditions, and (4) exposure to STM in light and darkness.

Methods. The organism used for most of these studies is classified as *Euglena gracilis* var. *bacillaris*.¹ It has the advantage over the widely used Pringsheim strain of growing rapidly up to 32°C, while the Pringsheim strain does not grow above 28°C. Illumination for growth was supplied by 3500°K white or 6500°K "daylight" fluorescent lamps, at intensity levels of 150 to 300 foot candles. This was sufficient for practical light saturation of moderately dense cultures.

Streptomycin solutions were compounded from crystalline salts and were sterilized by filtration through sintered glass.

It has been found previously in unpublished

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¹ Pringsheim, E. G., personal communication.

experiments that *E. gracilis*, and the other green and colorless euglenoids available in

TABLE I.

Basal Medium.	
	Concentration (%)
KH ₂ PO ₄	.1
K ₂ HPO ₄	.1
NH ₄ NO ₃	.1
MgSO ₄ · 7H ₂ O	.02
K ₂ citrate · H ₂ O	.08

Liver concentrate (Lederle, 1 ml \approx 3.3 U.S.P. units), 0.02 ml/100 ml.

Trace elements (mg %): Ca 2.0, Fe 0.3, B 0.02, Co 0.01, Cu 0.001, Mn 0.1, Mo 0.01, V 0.001, Zn 0.02.

pH 6.8-7.0. Media distributed 10 ml/25 ml Erlenmeyer flask.

Notes: 1. Citrate, added as a metal carrier,² is not utilized by *E. gracilis*.

2. The organism grows through a remarkably wide pH range, even in the presence of acetate or 0.25% Na butyrate. In later work the initial pH was 6.5.

3. Liver concentrates from 2 other commercial sources gave similar results.

supplemented with good substrates, gives green cells only a slight advantage in light over colorless cells.

Results. The effect of STM as influenced by concentration, substrates, and illumination was observed in the experiment summarized in Table II, using the medium of Table I. The observations on color refer to the macroscopic aspect of the cultures. *E. gracilis* strains normally become colorless when placed in darkness and become green again rapidly when restored to light. The following conclusions were drawn from the experiment of Table II and similar experiments:

1. STM reduced growth in light to a level equal to or lower than that in darkness.

2. Growth in darkness was unaffected. As a corollary, STM did not interfere with the utilization of externally supplied substrates.

3. Certain amino acids (alanine and glutamate) were good substrates for growth in

TABLE II.

Effect of Varying Concentrations of Streptomycin (STM) and of Various Substrates in Light and in Darkness. Growth expressed as cells/mm²

	Light				Darkness			
	No STM	STM 100 μ g/ml	STM 1000 μ g/ml	STM 5000 μ g/ml	No STM	STM 100 μ g/ml	STM 1000 μ g/ml	STM 5000 μ g/ml
	G	G	W					
No substrate	1300	410	8	0	8	10	13	51
Na butyrate	G	W	W	W				
0.15%	1900	760	480	238	950	680	720	880
Na H glutamate	G	G	W	W				
0.3%	1950	750	13	43	204	178	190	189
DL-Alanine	G	G	W	W				
0.25%	980	300	190	132	360	420	320	410
Glucose	G	G	W	W				
1.0%	1380	240	24	42	430	158	147	147

W—white, G—green. All cells in darkness were white.

pure culture, were stimulated several hundred-fold by, or required absolutely, an unidentified growth factor present in relatively very high concentration in liver concentrates refined for parenteral treatment of pernicious anemia. It was thenceforth simple to devise reproducible media virtually devoid of utilizable carbon and energy sources ("substrate"), but which allowed very heavy growth in light or darkness when appropriate substrates were added. A medium used in initial studies of bleaching is shown in Table I. This medium,

darkness, although they accelerated growth in light only slightly; they were inferior however to certain lower fatty acids (represented in Table II by butyrate).

In later experiments, a comparison was made of the action of STM upon cells grown under conditions permitting active proliferation as contrasted with non-proliferating cells. To cope with the possibility that STM-treated cells might acquire additional nutritional requirements, or that STM-dependent individuals might arise, the experiments were designed as follows: (a) Proliferating conditions were obtained by using a medium containing

² Hutner, S. H., *Trans. N. Y. Acad. Sci., Ser. II*, 1948, 10, 136.

butyrate, glutamate, tryptic digest of casein (Sheffield's "N-Z-Case") 0.3%, solubilized liver (Wilson's fraction "L") 0.02%, and yeast extract (Difco) 0.04%. This portion of the experiment was conducted in light and darkness with varying time of exposure to a constant concentration of STM (100 $\mu\text{g}/\text{ml}$), and with varying concentrations of STM with constant time of treatment (15 days). Flasks were inoculated with one drop of a normal green culture. At the end of the experimental periods, 1-drop transfers were made into duplicate flasks containing the same medium, one set having 100 $\mu\text{g}/\text{ml}$ STM, the other set without STM. The original cultures and the transfer cultures were then incubated in light for observation of the degree of bleaching. (b) Exposure to STM under non-proliferating conditions was obtained by first growing the cultures in light for 2 weeks in a substrate-free medium similar to that in Table I, thus making the cells wholly dependent on photosynthesis for energy and carbon. The cultures were then moved into darkness, and, after a 24-hour period to allow the dissipation of reserve food, treatment with STM was begun and carried out in darkness. At the end of the periods of exposure, 1-drop seedings were made as before into the complex medium, again with and without STM, and the cultures incubated in light. The salient observations from these experiments were:

1. There was a smoothly progressive bleaching of the cultures with increasing duration of exposure and increasing concentration of STM. The secondary cultures in STM-free media accurately reproduced the gross aspect which the primary culture had at the moment of transfer, if a small allowance be made for the slight selective advantage of the green cells in light.

2. There was no difference in the degree of bleaching between proliferating and non-proliferating cultures, either in respect to the necessary duration of exposure to STM or to the concentration of STM required. There was no difference in these respects between light- and dark-treated cultures under proliferating conditions.

3. There was no evidence of development

of STM-dependency, even in cultures exposed for 2 weeks to 1000 $\mu\text{g}/\text{ml}$.

4. With STM constant at 100 $\mu\text{g}/\text{ml}$, an exposure of 1 to 8 hours was required for loss of roughly 50% of the color, and 4 or more days for apparently complete bleaching.

5. With constant time of exposure (15 days), 1 $\mu\text{g}/\text{ml}$ gave roughly 50% loss of color, and bleaching was nearly complete with 40 $\mu\text{g}/\text{ml}$.

6. Preliminary microscopic examination of partly bleached mass cultures showed them to consist of both pale and of almost fully green individuals. However a few green cells had only 1-2 somewhat ill-defined chloroplasts instead of the normal number (about 10). Also, some green cells did not have chloroplasts of normal thickness. Aged white cells, derived both from primary treated cultures and from subcultures, accumulated an unusually large number of red granules as compared with aged untreated green cells or with cells of *Astasia*. The stigma and paramylon grains remained permanently unaltered in all colorless cultures.

Similar experiments on a restricted scale were carried out with *E. gracilis* var. *urophora* and the classical Pringsheim strain. Bleached cells of *E. gracilis* var. *urophora* have now been carried through 5 transfers in light on STM-free media, and *E. gracilis* var. *bacillaris* through 9, and the Pringsheim strain through 2, without the least resumption of color. Parallel tests on several species of *Astasia* showed that like *E. gracilis* in the dark they were not inhibited by 5000 $\mu\text{g}/\text{ml}$ STM, the highest concentration tested.

Discussion. "Directed evolution" has so far been achieved only in bacteria, as in the interconversion of types of *Pneumococcus*³ and of *Escherichia coli*⁴ under the influence of specific nucleoproteins. The change to the non-chlorophyll condition (apochlorosis) described here falls rather into the special class of "loss mutations" ("pertes de fonctions")

³ McCarty, M., Taylor, H. E., and Avery, O. T., *Cold Spring Harbor Symposia Quant. Biol.*, 1946, **11**, 177.

⁴ Boivin, A., *Cold Spring Harbor Symposia Quant. Biol.*, 1946, **12**, 7.

discussed by Lwoff.⁵ That this change follows a well-established evolutionary path is suggested by the following considerations:

1. Permanently colorless euglenoids similar to *E. gracilis* are abundant in nature, and are referred to the genus *Astasia*.⁶ Some species of *Astasia* have a stigma like that in the colorless strains here derived from *E. gracilis*.

2. The euglenoids, as compared with other algae, have one of the richest arrays of colorless counterparts of green species.⁵

3. As already mentioned, the strains of *E. gracilis* tested all grow well in darkness when provided with suitable substrates in a good basal medium and permitted free access to atmospheric oxygen, indicating that the loss of photosynthesis does not here involve a profound alteration in metabolism.

4. Colorless euglenas have been reported to arise spontaneously in artificial culture (see^{5,6} for references).

Sexuality is lacking in euglenoids and therefore it is impossible to determine here by the methods used for higher plants whether bleaching is a purely cytoplasmic phenomenon or involves a certain measure of conventional genic control—the classic problem of plastid autonomy. Another related question is whether bleaching, permanent or temporary, can be induced in forms which retain chlorophyll when grown in the dark.

In photosynthetic bacteria, as in blue-green algae, chlorophyll is not localized in plastids. Strains of purple bacteria (*Athiorhodaceae*) adapted to aerobic conditions, grow well in

darkness aerobically, with retention of characteristic color, although the pigments are diminished in amount.⁷ When 5 strains of purple bacteria, representing 5 species, were treated with STM, the sharp inhibition of growth was the same in light and darkness, and there were no obvious changes in color in partially inhibited cultures.

During the preparation of this paper, articles by von Euler and Bracco⁸ were received. They found that seeds germinated in STM solutions developed into seedlings whose leaves showed chlorophyll deficiencies associated with colorless or structurally defective plastids. They did not report observations beyond the seedling stage.

The effectiveness in respect to *Euglena*-bleaching of portions of the STM molecule, and of other antibiotics, actinomycete and non-actinomycete in origin, is being studied; such data should determine whether the bleaching phenomenon may be used as a specific test for streptomycin, and should also furnish some indication as to the mode of action of STM.

Summary. When acted upon by streptomycin, certain strains of *Euglena gracilis* undergo a permanent loss of chlorophyll, without being inhibited in growth if good energy and carbon sources are supplied to make up for the loss of photosynthesis. The degree of bleaching is proportional to the degree of exposure to streptomycin. Proliferating and non-proliferating cells, and cells exposed in the light and in the dark, appear equally susceptible to bleaching.

⁵ Lwoff, A., *L'Évolution Physiologique. Étude des Pertes de Fonctions Chez les Microorganismes*. Paris: Hermann et Cie, 1943, pp. 308.

⁶ Pringsheim, E. G., *Biol. Rev. Camb.*, 1948, **23**, 46.

⁷ van Niel, C. B., *Bact. Rev.*, 1944, **8**, 1.

⁸ Euler, H. v., *Archiv f. Kem., Min., Geol.*, 1948, **25A**, 1; Bracco, M., and Euler, H. v., *Kem. Arb. II*, 1948, **10**, 1; Bracco, M., and Euler, H. v., *Kem. Arb. II*, 1947, **10**, 1.

Absorption of Aortic Atherosclerosis by Choline Feeding.*

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A previous report by the authors described the preventive action of choline against the development of atherosclerosis of the aorta in a series of rabbits fed cholesterol and choline simultaneously.¹ Steiner² found highly suggestive evidence that 0.5 g of choline daily caused some reabsorption of atheromatous lesions produced in the aortas of 6 rabbits by cholesterol feeding for 170 days. In 4 other rabbits similarly treated by Steiner, choline failed to cause significant aortic atheromatous reabsorption. The present study was undertaken to determine whether choline could effect reabsorption of aortic atheromatosis in a larger series of rabbits fed double the dose of choline heretofore employed and studied over twice as long a period of time.

Anitschkow,³ Scarff,⁴ and other have demonstrated that the characteristic atheromatosis of the aorta does not disappear after withdrawing cholesterol from the diet of rabbits. This atherosclerosis persists up to 815 days after cholesterol withdrawal from the diet, indicating that spontaneous reabsorption of the aorta does not occur.

Forty-four white male rabbits approximately 3 months old, who were litter mates were used in this study. These animals were divided into Groups A and B each containing

21 and 23 animals respectively. All the animals were individually fed 0.5 g of cholesterol daily with a basic diet of Purina Chow for 184 days. The cholesterol was mixed with the chow as prepared especially for this study by the Golden State Milling Co. of Downey, Calif. The rabbits took their feedings without difficulty and showed a gain in weight in each instance.

At the end of the 184-day cholesterol feeding period and the cholesterol feedings were stopped in all animals. The regular chow diet was then given to the 21 Group A animals for 185 days; the animals were then sacrificed. After the discontinuation of the cholesterol feedings 23 Group B animals were fed 1.0 g choline daily for 185 days mixed with the chow as above. The animals took these feedings without difficulty, gaining weight and showing normal activity in all cases. At the end of the 185-day period the animals were sacrificed.

At postmortem examination the grade of aortic atherosclerosis was described as 0 whenever no involvement of the aorta by atherosclerosis was grossly visualized or palpated by the examining fingers. Grade 1 was employed to describe a slight degree of atherosclerotic involvement of the intima of the aorta. Grade 2 described moderate atherosclerosis of the aorta. Grade 3 indicated marked atherosclerosis. Grade 4 described complete atherosclerotic involvement of the surface area of the aorta.

Table I, Group A—the 21 control rabbits fed cholesterol alone for 182 days then the regular diet for 185 days showed at autopsy aortic atherosclerosis of varying degree in each animal.

Table I, Group B—the 23 rabbits fed cholesterol alone for 182 days, then the regular diet with 1.0 g choline daily for 185 days showed aortic atherosclerosis at autopsy

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¹ Morrison, L. M., and Rossi, A., to be published.

² Steiner, A., *Proc. Soc. Exp. Biol. and Med.*, 1938, 39, 411.

³ Anitschkow, N., *Verhandl. d. deutsch. path. Gesellsch.*, 1928, 23, 473.

⁴ Scarff, R. W., *J. Path. and Bact.*, 1927, 30, 647.

TABLE I.
Atherosclerotic Involvement of the Aorta in Rabbits Fed 0.5 g Cholesterol Daily for 184 Days.

Grade of involvement	0	1	2	3	4	Total affected
No. of rabbits	0	2	3	5	11	21
% of rabbits affected	0	9%	14%	24%	53%	100%

TABLE II.
Atherosclerotic Involvement of the Aorta in Rabbits Fed 0.5 g Cholesterol Daily for 184 Days,
Then 1.0 g Choline Daily for 185 Days.

Grade of involvement	0	1	2	3	4	Total affected
No. of rabbits	17	4	2	0	0	23
% of rabbits affected	74%	17%	9%	0	0	26%

in 26% of animals. Seventy-four percent of animals were free of demonstrable aortic atherosclerosis.

Conclusion. Choline caused reabsorption

of aortic atherosclerosis in the majority of rabbits whose lesions had been produced by cholesterol feeding.

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The Relationship of Heparin Activity to Platelet Concentration.

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(Introduced by G. S. Mirick.)

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An increased susceptibility of thrombocytopenic blood to heparin has recently been reported by Allen and his associates.¹ These authors found that following the addition of a standard concentration of heparin to blood, more protamine sulfate was necessary to restore the normal clotting time in thrombocytopenic blood than was required by normal controls. They interpreted this observation as suggesting that in thrombocytopenia an increased amount of a heparin-like substance may be present in the blood. Studies in our laboratory² on 12 patients with hemorrhagic diathesis associated with thrombocytopenia in no instance revealed evidence of a circulating anticoagulant. It therefore seemed quite plausible that the increased susceptibility of thrombocytopenic blood to heparin may be

a direct result of the decreased number of platelets present.

Experiments. Using silicone-treated apparatus and high speed centrifugation (12,000 to 14,000 RPM, 17,500 to 22,000 g's) at low temperature (4°C), platelet-free plasma was obtained from normal individuals without the use of anticoagulants. This plasma could be kept fluid in iced silicone-treated tubes for at least several hours. Platelet-rich plasmas were obtained by a similar technic except that the blood was centrifuged only at 3000 RPM (about 1300 g) for 5 minutes. Platelet counts were performed on these plasmas. These two plasmas were mixed in different proportions and varying amounts of heparin* added. Clotting times of these plasmas in glass tubes were then determined using a 3 tube method at 37°C. Results are shown in the table.

These results clearly indicate that the

¹ Allen, J. G., Bogardus, G., Jacobson, L. O., and Spurr, C. L., *Ann. Int. Med.*, 1947, **27**, 382.

² Conley, C. L., Hartmann, R. C., and Morse, W. I., II, *Bull. Johns Hopkins Hosp.*, in press.

* Solution of heparin (sodium salt), Lederle Laboratories, Inc.

TABLE I.
Experiment 1.

Clotting Times of Plasma Samples Containing Varying Numbers of Platelets and Concentrations of Heparin as Indicated. The clotting times were determined simultaneously on samples derived from pooled plasma specimens. Clotting times of each of the 3 tubes are recorded.

Clotting times in minutes of 1.0 ml portions in glass tubes at 37°C					
Heparin (mg per ml of plasma)	Whole blood (Platelets 190,000 per cmm)	"Platelet-rich" plasma (Platelets 420,000 per cmm)	"Platelet-free" plasma 90% "Platelet-rich" plasma 10% (Platelets 42,000 per cmm)	"Platelet-free" plasma 95% "Platelet-rich" plasma 5% (Platelets 21,000 per cmm)	"Platelet-free" plasma (Platelets 2 per cmm)
0	(1) 14 (2) 15 (3) 16	(1) 12 (2) 12 (3) 14	(1) 12 (2) 16 (3) 16	(1) 12 (2) 18 (3) 18	(1) 16 (2) 16 (3) 20
0.00025	(1) — (2) — (3) —	(1) — (2) — (3) —	(1) — (2) — (3) —	(1) 10 (2) 24 (3) 28	(1) 44 (2) 78 (3) Partial clot in 24 hrs.
0.0005	(1) — (2) — (3) —	(1) — (2) — (3) —	(1) 12 (2) 30 (3) 36	(1) 60 (2) 89 (3) 98	(1) No clots (2) in 24 hrs (3)
0.001	(1) 15 (2) 16 (3) 20	(1) 15 (2) 16 (3) 20	(1) 32 (2) 53 (3) 66	(1) 38 (2) Partial (3) clots in 24 hrs	(1) No clots (2) in 24 hrs (3)
0.01	(1) 7 hrs (2) No clots (3) in 24 hrs	(1) 51 (2) 113 (3) Partial clot in 24 hrs.	(1) No clots (2) in 24 hrs (3)	(1) No clots (2) in 24 hrs (3)	(1) No clots (2) in 24 hrs (3)

Experiment 2.

	Whole blood (Platelets 210,000 per cmm)	"Platelet-rich" plasma (Platelets 448,000 per cmm)	"Platelet-free" plasma 90% "Platelet-rich" plasma 10% (Platelets 44,800 per cmm)	"Platelet-free" plasma 95% "Platelet-rich" plasma 5% (Platelets 22,400 per cmm)	"Platelet-free" plasma (Platelets 4 per cmm)
0	(1) 11 (2) 15 (3) 16	(1) 4 (2) 8 (3) 10	(1) 10 (2) 14 (3) 17	(1) 10 (2) 14 (3) 16	(1) 10 (2) 16 (3) 17
0.00025	(1) — (2) — (3) —	(1) 10 (2) 14 (3) 24	(1) 10 (2) 14 (3) 21	(1) 19 (2) 37 (3) 60	(1) 23 (2) Partial clots (3) in 24 hrs
0.0005	(1) — (2) — (3) —	(1) 11 (2) 20 (3) 28	(1) 23 (2) 26 (3) 31	(1) 14 (2) 35 (3) 56	(1) Partial clots (2) in 24 hrs (3) No clot in 24 hrs
0.001	(1) 22 (2) 24 (3) 29	(1) 11 (2) 22 (3) 26	(1) 19 (2) 54 (3) 65	(1) 41 (2) 65 (3) 90	(1) Partial clot in 24 hrs (2) No clots in (3) 24 hrs
0.01	(1) 60 (2) No clots (3) in 24 hrs	(1) 41 (2) 93 (3) 138	(1) No clots (2) in (3) 24 hrs	(1) No clots (2) in (3) 24 hrs	(1) No clots (2) in (3) 24 hrs

HEPARIN ACTIVITY AND PLATELET CONCENTRATION

TABLE I (Continued).
Experiment 3.

Heparin (mg per ml of plasma)	Clotting times in minutes of 1.0 ml portions in glass tubes at 37°C			
	"Platelet-rich" plasma (Platelets 208,000 per cmm)	"Platelet-rich" plasma 75% (Platelets 156,000 per cmm)	"Platelet-free" plasma 50% "Platelet-rich" (Platelets 104,000 per cmm)	"Platelet-free" plasma (Platelets 8 per cmm)
0	(1) 5 (2) 6 (3) 7	(1) 7 (2) 9 (3) 9	(1) 5 (2) 9 (3) 12	(1) 7 (2) 13 (3) 21
0.001	(1) 14 (2) 24 (3) 32	(1) 12 (2) 20 (3) 29	(1) 16 (2) 27 (3) 41	(1) 25 (2) 67 (3) 91
0.005	(1) 24 (2) 52 (3) 81	(1) 25 (2) 73 (3) 100	(1) 36 (2) 52 (3) 83	(1) No clots (2) in (3) 3 hrs
0.01	(1) 36 (2) 74 (3) 105	(1) 44 (2) 108 (3) No clot in 3 hrs	(1) 91 (2) No clots (3) in 3 hrs	(1) No clots (2) in (3) 3 hrs

delaying or inhibitory action of heparin on the clotting of normal plasma is inversely related to the concentration of platelets present.

Discussion. The interpretations of several clinical tests for detecting hypo- and hypercoagulability of blood have been based on rather uncertain theoretical grounds. One of these is the test devised by Allen and his associates¹ for determining the existence of an increased amount of a heparin-like substance in thrombocytopenic blood. Our observations show that the results of their tests can be explained by the variation in the platelet concentration alone.

The "heparin tolerance test"³ has been proposed as a means of measuring the tendency to intravascular clotting. Although this test has been used in clinical studies, the variables

which determine its results have been to a large extent unknown. Our experiments suggest that one factor which may influence the results of this test is the number of platelets present.

In tests in which there is a minimal agitation of normal platelet-free plasma, the addition *in vitro* of heparin to a final concentration of 0.0005 mg per ml completely inhibits coagulation in glass tubes at 37°C. Normal platelet-free plasma to which no anticoagulant has been added invariably clots when transferred to glass tubes at 37°C. It therefore seems reasonable that the amount of active heparin present in normal plasma is of the order of magnitude of 0.0005 mg per ml or less.

Conclusions. 1. The concentration of heparin required to inhibit or delay coagulation is directly related to the number of platelets present.

³ de Takats, G., *Surg., Gyn., and Obs.*, 1943, **77**, 31.

2. The increased susceptibility of thrombocytopenic blood to heparin is a direct manifestation of the decreased number of platelets present and does not necessarily indicate the presence of a heparin-like substance.

3. One variable which may influence the

results of the "heparin tolerance test" is the number of platelets present.

4. Our results suggest that the amount of active heparin present in normal plasma is very small and at least of the order of magnitude of 0.0005 mg per ml or less.

16694 P

Effects of Electro-Convulsive Shock on Pregnancy in the Rat.*

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In an investigation of the effects of electro-convulsive shock on the maternal behavior of the white rat, Rosvold¹ found that females did not bear litters if shocked within 12 to 15 hours after copulation and once daily for 14 days thereafter. The rats received 45 milliamperes for 0.2 second through alligator clips attached to the ears. This strength of current invariably induced a complete tonic-clonic convulsion.

Gross examination at autopsy suggested that the fetuses were being resorbed. No histological study of the uteri, ovaries, or pituitaries was made in the original experiments. Accordingly a series of experiments was repeated in order to permit microscopic study of these organs under various conditions with respect to the number of shocks and the time of their administration. This preliminary report is based on the study of 24 healthy nulliparous females, about 100 days of age in which mating was observed in the evening and vaginal plugs found on the following morning. The animals were divided into 3 groups and shocked according to the following schedules:

Group I. Five animals shocked once, 12 hours after mating. Three controls mated at the same time, but not shocked.

Group II. Five animals shocked once daily for 6 days beginning 12 hours after mating. Three controls mated at the same time, but not shocked.

Group III. Five animals shocked once daily for 11 days beginning 12 hours after mating. Three controls mated at the same time, but not shocked.

All animals were sacrificed with ether on the eleventh day after copulation and the uteri, ovaries, and pituitaries removed at once and fixed in Bouin's fluid. Table I indicates the results observed at autopsy. The uteri of all the controls contained normal embryos of the 11th day of development.

In the two uteri of Group II which showed resorption of the embryos, the remnants of the implantation sites were located on the antimesometrial side of the uterus. In all of the animals of Group III the implantation sites were located on the mesometrial side of the uterus and were reduced to from 1/2 to 1/5 the size of those in the uteri of control animals.

For this preliminary study a randomly selected implantation site from each animal was sectioned serially at 10 μ and stained with hematoxylin and eosin. Sections of ovaries were stained with hematoxylin and eosin, and pituitaries with Mallory-azan.

In the uteri of the two animals of Group II in which the conceptuses were undergoing resorption, the deciduae were apparently normal and contained many giant cells, but

* Supported by a grant from the Scottish Rite Fund, administered at Stanford University by Calvin P. Stone.

¹ Rosvold, H. E., Doctoral dissertation, Stanford University, 1948.

TABLE I.
Effects of Electric Shock on Rats Autopsied on the 11th Day of Pregnancy.

Group No.	No. of animals	No. of shocks	Uterine contents	
			Normal	Resorbing
I	5	1	5	0
II	5	6	3	2
III	5	11	0	5

Control animals are not included.

appeared in a condition to be expected at 7 or 8 days of pregnancy rather than at 11 days. Some proliferation was occurring as indicated by the presence of a few mitotic figures in each section. The embryos, however, were similar in stage of development to normal embryos of the 6th day but were disintegrating. In the uteri of all animals of Group III the entire decidua was disintegrating and no embryonic tissue could be found. The uterine lining was being reconstituted so as to expel the entire necrotic mass into the lumen. The uteri of the 3 unaffected animals contained normal embryos.

The pituitaries of all of the affected animals in Group II and III showed some degranulation of the acidophils and, although no measurements were made, the basophils appeared reduced in size when compared with those of the controls. The pituitaries of the unaffected animals did not show these

changes. The ovaries of all the affected animals showed many degenerating corpora lutea and a few which were histologically normal, but smaller than those of controls. The ovaries of the unaffected animals were essentially normal.

Summary. A single electro-convulsive shock administered to 5 pregnant rats 12 hours after mating had no effect on the course of pregnancy. Of 5 rats shocked once daily for 6 days beginning 12 hours after copulation, on the eleventh day 2 showed some degranulation of pituitary acidophils, slight reduction in size of basophils, small or degenerating corpora lutea and degenerating retarded embryos in the uteri. All animals similarly shocked for 11 days, showed the same pituitary and ovarian changes, and, in addition, complete destruction of the fetuses with necrosis and sloughing of decidua into the uterine lumen.

16695

Effects of Acute Intermittent Anoxia Upon Urinary Volume, Specific Gravity and Chloride.

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(Introduced by J. P. Quigley.)

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Silvette¹ demonstrated a 300% increase in urinary volume of rats exposed to 282 mm Hg barometric pressure for 3-hour periods. Ten human subjects exposed by Armstrong²

to simulated 12,000 ft. for 4 and 7 hours in a low pressure chamber excreted 100 to 300% more urine than at sea level and the urinary specific gravity fell as low as 1.016. Pincus and Hoagland³ observed a polyuria in men exposed to reduced oxygen pressure. Burrill,

¹ Silvette, H., *Am. J. Physiol.*, 1943, 140, 374.
² Armstrong, H. G., *Principles and Practice of Aviation Medicine*, The Williams and Wilkins Co., Baltimore, 1939, p. 284.

³ Pincus, G., and Hoagland, H., *J. Aviation Med.*, 1943, 14, 173.

Freeman and Ivy⁴ detected a slight increase in urinary volume and chloride content in men exposed to 18,000 ft. for 2-hour periods. Langley and Clarke⁵ and Lewis *et al.*⁶ reported a polyuria in unanesthetized dogs exposed for prolonged periods to 18,000 and 20,000 ft. altitude equivalents.

The present investigation was undertaken to determine the effects of severe anoxic conditions upon urinary volume, specific gravity and chloride excretion at various stages of the anoxic period. In the work of earlier investigators anoxic exposures have been made over several hours and in such experiments the initial urinary changes have been masked by changes of a different character occurring during a latter portion of the anoxic period.

Methods. Adult Long-Evans rats, male and female, were exposed to reduced barometric pressures in chambers described by Van Middlesworth *et al.*⁷ and urine collections were made after the method of Silvette.¹ All exposures to reduced pressure were made at 258 mm Hg (equivalent 27,000 ft.) at 23-26°C. The method differed from those of earlier investigators in that samples were collected every hour. This necessitated returning the animals to sea level pressures for 3 minutes at the end of each hour, returning the animals to the chambers, and redecompressing for another hour. This procedure was repeated until an anoxic interval of 3-5 hours had accumulated.

Urinary specific gravity was determined by the falling drop method, in which a standard drop was allowed to fall through a 124 cm column of benzene and the falling time (range 11.0-13.0 sec.) was measured to within 0.02 sec. Chloride analyses were made on 1 cc of urine by means of the Volhard-Harvey technic.⁸ Urine collections from control animals were made over 4-6 hour periods, and

the average excretion per hour was taken as the control value. All animals were fed *ad libitum*, but water was usually withheld for 2 hours before each experiment. The manipulations associated with the changing of the environmental pressure failed to modify the urine volume in 12 control animals subjected to the reduced barometric pressure (258 mm Hg) for 2 minutes before and after the control period. The urine volumes were essentially the same (0.10-0.16 cc/100 g/hr) as those animals simply allowed to remain continuously at sea level. No rats were used more than once in a 4 week period; thus the possibility of acclimatization was avoided. Over 100 rats were used throughout the investigation.

Results. One hour exposure to 258 mm Hg barometric pressure resulted in a drastic increase in urinary output (Fig. 1A). This increase ranged usually from 3-11 times the normal value. Temperatures around 24°C and adult female rats usually tended to produce the greatest volume of urine although occasionally female animals exhibited minimal effects. Young adult male animals often produced only the lower volumes. After the polyuria of the first hour, the rate of urinary excretion decreased but still averaged above normal for the duration of the experiment.

The specific gravity of the abundant first hour urine usually ranged from 1.004 to 1.010 (Fig. 1B). After the first hour, the specific gravity also began to approach control values but did not attain the original level during the experimental period.

Urinary chloride was greatest in the most dilute, most abundant urine (Fig. 1C) and the chloride concentration was drastically reduced as the total solid content increased with continued anoxia. After 3 hours at these severe hypoxic conditions, the detectable chloride had almost disappeared from the urine.

One series of experiments was designed to determine the effect of the dehydration of the first hour exposure. Four groups of 3 rats each were given a saline solution at the end of each hour of altitude exposure. The same volume and chloride content as had been excreted in the previous hour urine collection

¹ Burrill, M. W., Freeman, S., and Ivy, A. C., *J. Biol. Chem.*, 1945, 157, 297.

⁵ Langley, L. L., and Clarke, R. W., *Fate J. Biol. and Med.*, 1942, 14, 529.

⁶ Lewis, R. A., Thorn, G. W., Koepf, G. F., and Dorrance, S. S., *J. Clin. Invest.*, 1942, 21, 33.

⁷ Van Middlesworth, L., Kline, R. F., and Britton, S. W., *Am. J. Physiol.*, 1944, 140, 474.

⁸ Harvey, S. C., *Arch. Int. Med.*, 1910, 6, 12.

URINARY CHANGES IN ACUTE HYPOXIA

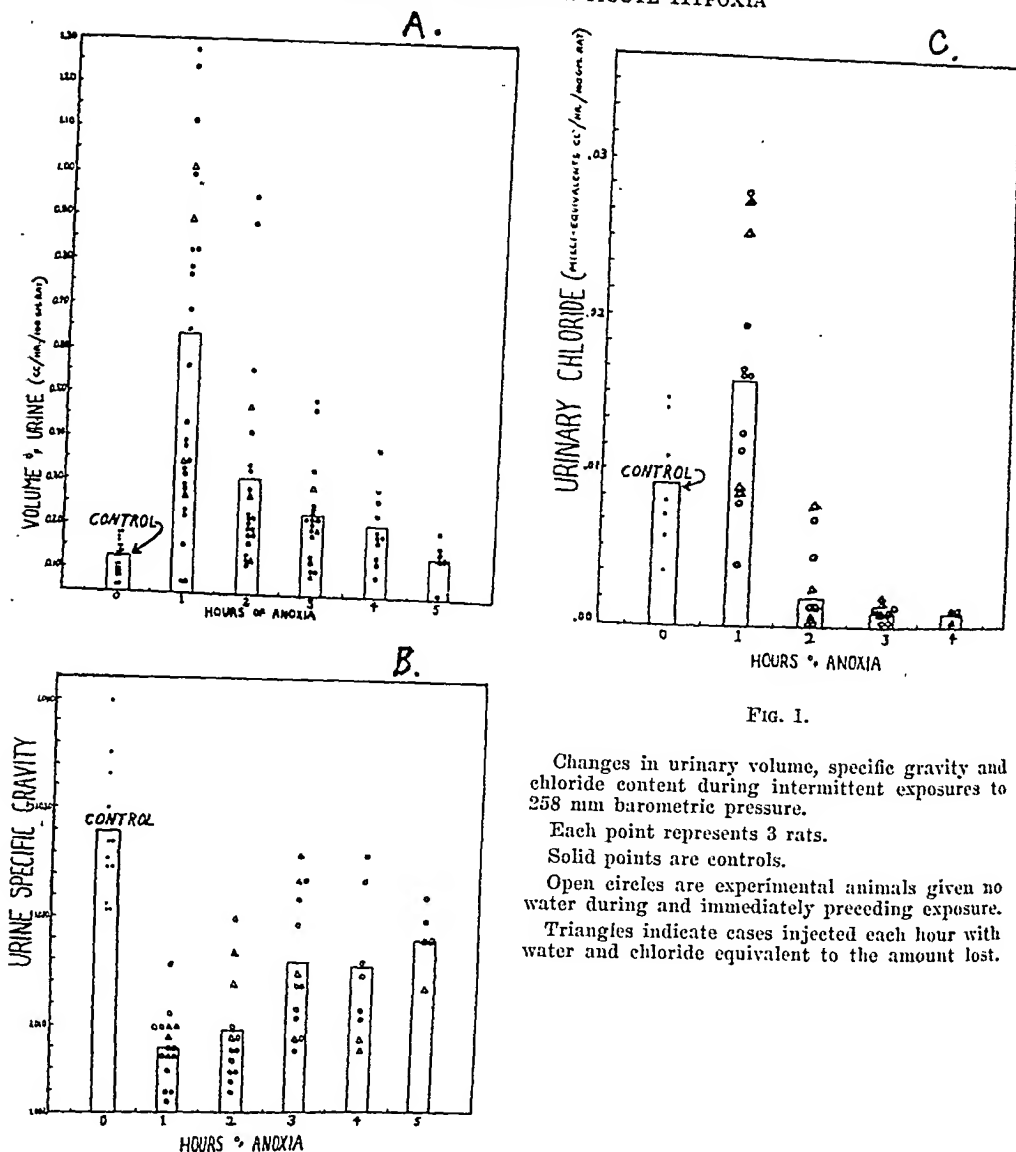


FIG. 1.

Changes in urinary volume, specific gravity and chloride content during intermittent exposures to 258 mm barometric pressure.

Each point represents 3 rats.

Solid points are controls.

Open circles are experimental animals given no water during and immediately preceding exposure.

Triangles indicate cases injected each hour with water and chloride equivalent to the amount lost.

was injected intraperitoneally. The subsequent urine volumes, specific gravity and chloride analyses are within the same ranges as the uninjected rats (triangle values in Fig. 1.) Apparently more comprehensive analyses for additional urinary components will be necessary for interpretation of the changes observed in urinary excretion during hypoxia.

Summary. The first hour exposure of rats to 258 mm Hg barometric pressure resulted in a 3 to 11 (average 7) fold increase in urine

production. This increase in volume was accompanied by a marked fall in specific gravity (1.004 to 1.012, average 1.008). At the end of the third hour of intermittent anoxia the urinary volume and specific gravity approached normal sea level values. The chloride content, on the other hand, was *greatest* in the very dilute and abundant urine of the first hour and the chloride content decreased on repeated exposures until the more concentrated urine of the third hour contained practically no chloride.

Influence of Saliva upon Hemagglutination by Influenza Virus.*

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Study of the mechanisms involved in infection and resistance to influenza virus led to the demonstration of a virus-inactivating property in nasal secretions and in sputum.^{1,2} The bulk of evidence points to the probability that the essential agency is specific antibody.³ Rose⁴ has continued further studies of human sputum, demonstrating wide variation in the capacity of different samples to neutralize influenza virus.

Recently several authors⁵⁻⁸ have described the inhibition of the hemagglutinating action of influenza virus by carbohydrates and viscous materials. Although in an earlier study² saliva was not found to neutralize influenza virus in mice, the present study was undertaken to determine the effect of saliva upon hemagglutination since that fluid commonly contains a variety of substances similar in character to those shown to influence the action of virus upon erythrocytes *in vitro*.

Materials and Methods. *Collection and Treatment of Specimens.* Twenty to 25 cc of saliva were collected without chewing, in 50

cc graduated centrifuge tubes. Specimens were centrifuged at 2000-2500 r.p.m. for half an hour to remove solid particles and mucus because these substances were found to cause the chicken red cell suspensions to settle out on the bottom of the tubes in the same pattern as that seen with influenza virus and red cells. If supernatants were clear after centrifuging they were removed and used with no further treatment. However, when the specimens contained a great deal of mucus, further treatment was necessary. They were ground in mortars with alundum and centrifuged again. If still not clear and free of mucus, they were filtered through cotton and gauze. Even then small amounts of mucus remained in some specimens. In most cases the saliva was used the same day as collected.

Virus Strain. Allantoic fluid of the PR8 strain of influenza virus Type A which had 593 mouse passages and 71 egg passages was used in the majority of the experiments. In a few of the early experiments an eluate prepared from the 65th egg passage and allantoic fluid from the 70th egg passage of the same strain were used.

Red Cell Suspensions. From a stock 10% suspension of washed chicken erythrocytes, 0.25% suspensions were prepared daily. No cells more than 5 days old were used.

Experimental. 1. *Effect of saliva on PR8 strain of influenza virus.* Two-fold dilutions of virus were made in 0.25 cc volumes of physiological salt solution. To each dilution 0.25 cc of undiluted centrifuged saliva was added and mixed. This mixture stood at room temperature for 30-60 minutes before 0.5 cc of 0.25% chicken red cell suspension was added. Control titrations of virus and saliva alone were carried out under the same conditions.

* This investigation was conducted with the aid of the Commission on Influenza, Army Epidemiological Board, Office of the Surgeon General, United States Army, Washington, D.C.

¹ Burnet, F. M., Lush, D., and Jackson, A. V., *Brit. J. Exp. Path.*, 1939, **20**, 377.

² Francis, T., Jr., *Science*, 1940, **91**, 198.

³ Francis, T., Jr., The Harvey Lecture Series, 1941-42, **37**, 69.

⁴ Rose, Harry M., and Prince, Eleanora M., *J. Clin. Invest.*, 1948, **27**, 554.

⁵ Green, R. H., and Wooley, D. W., *J. Exp. Med.*, 1947, **80**, 55.

⁶ Friedewald, W. F., Miller, E. S., and Whatley, L. R., *J. Exp. Med.*, 1947, **80**, 65.

⁷ Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Nature*, 1947, **160**, 404.

⁸ Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, **87**, 411.

The effect of saliva on the hemagglutinating titer of the PR8 strain of influenza virus was to decrease it from 8 to 512 times. The amount of inhibitor in saliva varied from person to person as well as from day to day in the same individual. Results of experiments with saliva from one individual on 6 occasions are shown in Table I. The results suggest that some deterioration of the inhibiting substance occurs on standing, even at 4°C.

2. *Inhibition tests with dilutions of saliva.* An inhibition test was performed employing a procedure similar to that described by Salk.⁹ Two-fold dilutions of saliva were made directly in 0.5 cc of 0.25% red cell suspension. To the dilutions of saliva, 0.5 cc of a 1/1000 dilution of PR8 virus was added. This amount of virus constituted a final dilution of two agglutinating units in each tube.

This method of measuring inhibition by saliva, at first glance, did not seem to be as effective as that of the first experiments, owing perhaps to the fact that in the second type of test the saliva and virus do not stand together before the indicator (cell suspension) is added. Nevertheless, in terms of the hemagglutinating units inhibited in the two types of test the differences are not great. The results with several specimens are shown in Table II.

3. *Effect of saliva on chicken red cells.* From 3.5 to 4 cc of saliva were mixed with 8 cc of 0.25% chicken red cells and allowed to stand at room temperature for 30-60 minutes. The specimens were thoroughly shaken several times during that period. The cells were centrifuged and resuspended in 8 cc of fresh salt solution without washing. These cells were then added to a series of virus dilutions. A cell control of 0.5 cc salt solution and 0.5 cc of cells was included.

It was found that when a specimen of saliva caused spontaneous agglutination of red cells, the cells continued to agglutinate even though removed from the mixture and resuspended in fresh salt solution. In most cases treating cells with saliva had very little effect on the virus titrations. Thus the influence of the inhibitor appears to be upon the

TABLE I.
Effect of Saliva from One Individual Upon Hemagglutination by the PR8 Strain of Influenza Virus.

Specimen	Experiment	Date of test	Age of saliva, days	Final dilution of virus										Titer of virus	Aggl. units of virus inhibited		
				4	8	16	32	64	128	256	512	1024	2048			4096	8192
Control	0.5 cc virus dil. +	With all tests		+	+	+	+	+	+	+	+	+	+	+	+	+	+
E.M.	0.5 cc cell suspension																
	0.25 cc virus dil. +	1-22-48	0	+	+	+	+	+	+	+	+	+	+	+	+	4096	
	0.25 cc saliva +	27	0	+	+	+	+	+	+	+	+	+	+	+	+	8	256
		2-23	6	+	+	+	+	+	+	+	+	+	+	+	+	32	64
		3-2	1	+	+	+	+	+	+	+	+	+	+	+	+	512	4
	0.5 cc cell suspension	3	1	+	+	+	+	+	+	+	+	+	+	+	+	128	16
		5	0	+	+	+	+	+	+	+	+	+	+	+	+	256	8
				+	+	+	+	+	+	+	+	+	+	+	+	4	512
	+																
	= Agglutination.																
	0 = No agglutination.																

⁹ Salk, J. E., *J. Immunol.*, 1944, 49, 87.

TABLE II.
Effect of Saliva upon Hemagglutination of PR8 Strain of Influenza Virus as Indicated by the Inhibition Test.

Specimen	Date of test	Age of saliva in days	Inhibition titer of saliva	Aggl. units of virus inhibited
B.B.	2-23-48	6	< 4	0
	26	0	32	64
	3- 2	1	64	128
	3	1	32	64
	29	0	32	64
A.Z.	17	0	8	16
J.Q.	17	0	8	16
E.M.	2-23	6	< 4	0
	3- 2	1	< 4	0
	3	1	4	8
	5	0	32	64

TABLE III.
Lack of Effect of Saliva Upon the Agglutinability of Chicken Red Cells.

Specimen	Experiment	Date of test	Age of saliva in days	Titer of virus	Aggl. units of virus inhibited
Control	0.5 cc virus dil. + 0.5 cc normal cells	With all tests		4096	
B.B.	0.5 cc virus dil.	1-28-48	0	1024	2
	+	2-23	6	2048	1
	0.5 cc cells saliva-treated	26	0	2048	1
A.Z.	"	26	0	4096	0
J.Q.	"	28	0	4096	0
E.M.	"	22	0	256	8
	"	27	0	512	4
	"	2-23	6	2048	1

virus rather than upon the erythrocytes. The results shown in Table III illustrate the general lack of effect as well as the exceptional experience with saliva of E.M.

4. *Effect of heating saliva and virus.* Samples of saliva or virus were heated at 56°C for 30 minutes. All various combinations of the above tests were tried:

(a) Constant amount of heated saliva added to serial dilutions of unheated virus.

(b) Inhibition test—serial dilutions of heated saliva with constant amount of unheated virus.

(c) Unheated saliva added to heated virus.

(d) Inhibition test—unheated saliva with heated virus.

(e) Heated saliva added to heated virus.

(f) Inhibition test—heated saliva with heated virus.

One effect of heating the saliva was the elimination of spontaneous agglutination of red cells by certain specimens. In only one case did heating the saliva fail to eliminate this influence. Otherwise the heating of virus or saliva exerted no significant effect upon the action of saliva, illustrating that in this

respect the inhibitory influence of saliva differs from that of the normal serum inhibitor.

Summary. Saliva of human adults possesses the capacity to inhibit agglutination of erythrocytes by influenza virus. Under the conditions of study the effectiveness varies among individuals and at different times. Its action is not significantly affected by heating

at 56° C for 30 minutes; nor is its behavior influenced by heating of the virus. Nevertheless, the action of the inhibitor appears to be upon the virus rather than upon the erythrocytes. The nature of the inhibitor has not been determined but it is suggested that saliva represents a physiologic source of materials such as have been shown to interfere with hemagglutination in *in vitro* systems.

16697

Alteration of *Pasteurella pestis* Bacteriophage Following Successive Transfer on *Pasteurella pseudotuberculosis* and on Shigellae.

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A previous report¹ has described the activity of a strain of *Pasteurella pestis* bacteriophage which was able to lyse 19 of 27 strains of *Pasteurella pseudotuberculosis* and 6 of 37 strains of shigellae. By repeated transfer on *P. pseudotuberculosis* (Spokane strain) this phage became adapted so that it lysed all 27 strains of this species in high dilution. After transfer on shigellae, the phage increased its potency for sensitive strains of this genus. The present report describes the effect of successive transfer of the same *P. pestis* phage on *P. pseudotuberculosis* and on certain species of shigellae in various combinations.

The methods used in this study have been recorded.¹ All tests were incubated at 37°C. The transfers of phage were made by adding 0.5 ml of the previous passage to 10 ml of a broth culture of bacteria about two hours old and incubating until clear. After about 5 transfers no secondary resistant growth appeared and filtration of the lysate was unnecessary. The "purified" *P. pestis* phage previously described was used as a starting point in this study. The bacterial cultures

were the same as those studied in the first report. Thirteen additional strains of *P. pseudotuberculosis* were investigated, of which all but one were lysed by the original phage.

In the first experiment, the *P. pestis* phage was transferred 22 times on *P. pseudotuberculosis* (Spokane strain) and then five times on each of the following in succession: *S. dysenteriae* (No. 44), *S. paradysenteriae* (type 103 No. 12), *S. sonnei* (No. 6), and *S. ambigua* (No. 33). It was found that any significant change occurred within 5 transfers on a given culture and that further transfers up to 25 made no difference. After each series of transfers, the adapted phages were tested on agar plates against each of the organisms listed and against *P. pestis*. Representative results are shown in Table I.

The results after transfer on 2 or 3 of the species of shigellae were essentially the same as those after transfer on all 4 species. The right hand column in Table I represents the effect of successive transfer on all 4 shigellae. It is noteworthy that after passage first on *P. pseudotuberculosis* and then on *S. dysenteriae*, the phage no longer lysed *P. pseudotuberculosis*.

A second series of experiments was planned to show the effect of successive transfer on

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¹ Lazarus, A. S., and Gunnison, J. B., *J. Bact.*, 1947, 53, 705.

TABLE I.
Highest Dilution of Original and Adapted Phages Producing Lysis of Plate Cultures of Various Organisms.

Organism tested	Original <i>P. pestis</i> phage	Original <i>P. pestis</i> phage adapted to		
		PTB	PTB then <i>S. dysenteriae</i>	PBT then <i>S. dysenteriae</i> , <i>paradysenteriae</i> , <i>sonnei</i> , and <i>ambigua</i>
<i>P. pestis</i>	10 ⁻⁷	10 ⁻⁷	10 ⁻⁷	10 ⁻⁶
PTB	—	10 ⁻⁷	—	—
<i>S. dysenteriae</i>	10 ⁻³	100	10 ⁻⁶	10 ⁻⁵
<i>S. paradysenteriae</i>	10 ⁻²	100	10 ⁻⁴	10 ⁻⁴
<i>S. sonnei</i>	10 ⁻¹	100	10 ⁻²	10 ⁻⁵
<i>S. ambigua</i>	10 ⁻⁴	10 ⁻⁴	10 ⁻⁶	10 ⁻⁵

— = no lysis. PTB = *P. pseudotuberculosis* (Spokane).

TABLE II.
Lysis After Transfer of *P. pestis* Phage on Pasteurellae and Shigellae.

Phage transferred on	Tested on <i>P. pestis</i>	Agar plate cultures of		
		9 PTB strains not lysed by original phage	31 PTB strains lysed by original phage	Shigellae*
<i>P. pestis</i>	++	—	++	+
PTB (Spokane)	++	++	++	+
Shigellae*	++	—	++	++
PTB—then <i>S. sonnei</i> , <i>paradysenteriae</i> , or <i>ambigua</i>	++	++	++	++
PTB then <i>S. dysenteriae</i>	++	—	++	++
PTB then <i>S. dysenteriae</i> then PTB again	++	++	++	++

++ = lysed by phage in dilution of 10⁻³ or higher.

+ = lysed by phage in dilution lower than 10⁻³.

— = not lysed.

PTB = *P. pseudotuberculosis*.

* Includes *S. dysenteriae*, *S. paradysenteriae*, *S. sonnei*, and *S. ambigua*.

different organisms in altering the characteristics of the original phage. The plan and the results are summarized in Table II.

It was again noted that phage first adapted to *P. pseudotuberculosis* (Spokane) and then transferred on *S. dysenteriae* lost its ability to lyse *P. pseudotuberculosis* (Spokane). This adapted phage was then tested against all 40 strains of *P. pseudotuberculosis*. It failed to lyse any of the nine strains which were resistant to the original phage, but lysed all 31 strains sensitive to the original phage. Hence this phage adapted to the Spokane strain and then to *S. dysenteriae* behaved just like the parent *P. pestis* phage in its action on *P. pseudotuberculosis* cultures.

Transfer of phage adapted to the Spokane strain on shigellae other than *S. dysenteriae* did not change its ability to lyse all the *P.*

pseudotuberculosis strains. Therefore the loss of activity against certain *P. pseudotuberculosis* cultures was produced only by *S. dysenteriae*. The potency of this phage for all strains of *P. pseudotuberculosis* was completely restored by again transferring it on the Spokane culture after passage on *S. dysenteriae*. Furthermore, the original phage adapted first to shigellae, including *S. dysenteriae*, and then to *P. pseudotuberculosis* (Spokane) lysed both the shigellae and all strains of *P. pseudotuberculosis*. Therefore, it seemed that transfer of phage adapted to *P. pseudotuberculosis* on *S. dysenteriae* produced a selective blocking action resulting in loss of ability to lyse a group of cultures previously sensitive.

Tests were made to determine whether this blocking action might be due to metabolic

or disintegration products of *S. dysenteriae* which, of course, would be present in phage adapted to this organism. A lysate of *S. dysenteriae* was prepared from a broth culture by adding phage adapted first to *P. pseudotuberculosis* and then to *S. dysenteriae*. This lysate was mixed with equal parts of phage adapted to *P. pseudotuberculosis* and allowed to stand at 37°C for two hours. The mixture was then added to broth and agar cultures of *P. pseudotuberculosis* using the same technic as usual. There was complete lysis in all tests. Hence, the presence of a lysate of *S. dysenteriae* did not inhibit lysis of *P. pseudotuberculosis* by phage adapted to the latter.

The plaques produced by the original *P. pestis* phage on agar cultures of *P. pestis* were uniform in size and measured about 3 mm in diameter after 24 hours. On plates of *P. pseudotuberculosis* and of shigellae, the plaques formed by the original phage varied in diameter from 0.5 to 3 mm. Large and small plaques were isolated repeatedly in attempts to separate two strains or mutants of phage, but without success. Isolated plaques gave rise to both large and small plaques even after many subcultures. Successive transfer on four species of shigellae followed by passage on *P. pseudotuberculosis* did not alter the size of plaques. The plaque size for a given culture was no different with the various adapted phages than with the original phage.

No explanation can be given at present for the alteration of phage adapted to *P. pseudotuberculosis* by subsequent transfer on *S. dysenteriae* so that it longer lyses certain strains of pasteurellae. It seemed possible that the original phage might contain two components and that the one acting on these strains could not multiply in the presence of *S. dysenteriae*. However, this is unlikely because the activity toward *P. pseudotuberculosis* was readily restored by readaptation to the Spokane strain after many transfers on *S. dysenteriae*.

The variation in plaque size shown by the original phage suggests that mutant types might be present which might vary in their host range, although attempts to separate

such mutants were unsuccessful. Delbruck² has reported the occurrence of biochemical mutants of phage with differing requirements for cofactors for adsorption of the phage on the host bacteria. One of his mutants required tryptophan or its analogues and its adsorption was inhibited by indole. These substances are not concerned here for the medium used is rich in tryptophan and neither *P. pseudotuberculosis* nor *S. dysenteriae* produce indole. Lysis was not inhibited by the addition of lysates of *S. dysenteriae* to mixtures of *P. pseudotuberculosis* and its adapted phage.

Likewise there is no apparent explanation for the difference in behavior of those strains of *P. pseudotuberculosis* resembling *P. pestis* in their sensitivity to the phages and of those not resembling *P. pestis*. No correlation could be demonstrated between the sensitivity of the strains to the various phages and their microscopic morphology or their antigenic structure. The only detectable difference was that the majority of the strains behaving like *P. pestis* produced rough colonies while those of the other group were usually smooth.

Summary. Bacteriophage, originally recovered as a lytic agent for *P. pestis*, also lysed 31 of 40 strains of *P. pseudotuberculosis*. After adaptation to one of these (Spokane strain), it also lysed the remaining nine strains. When the adapted phage was transferred on *S. dysenteriae*, it reverted to its original activity; i.e., it again failed to lyse the nine strains of *P. pseudotuberculosis* which resisted the original phage. Activity for all strains was restored by transferring it on the Spokane strain. Lysates of *S. dysenteriae*, mixed with the adapted phage, caused no reversion to the original selective activity for only 31 strains of *P. pseudotuberculosis*. Transfers of the adapted phage on *S. paradyenteriae*, *S. sonnei*, *S. ambigua*, or on all of these species in succession, did not alter its ability to lyse all strains of *P. pseudotuberculosis*. The size of plaques did not change during transfers on either the shigellae or the pasteurellae used.

² Delbruck, M., *J. Bact.*, 1948, 56, 1.

Fate and Distribution of Penicillin in the Body. II. Duration of Blood Concentration and Chemotherapeutic Effectiveness.*

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The original concept that the continuous presence of penicillin in the blood is essential for therapeutic effectiveness has been substantially weakened since the clinical reports of Tillet, McCormack, and Cambier.^{1,2} These authors obtained excellent results in the treatment of lobar pneumonia on a regimen which gave detectable blood levels only intermittently. These findings were confirmed by the experimental studies of Zubrod,³ White, Baker and Jackson⁴ and Gibson.⁵ The implications of such observations were discussed by Marshall⁶ and Eagle.⁷

Our studies⁸ on the distribution of penicillin in the organism required an analysis of the relationship between duration of therapeutic effectiveness and "blood level" as determined by *in vitro* methods.

Material and Methods. Albino rats of 60 to 220 g body weight were injected intramuscularly with a repository preparation consisting of a suspension of crystalline potassium penicillin G in peanut oil containing 1 mg/cc epinephrine.⁹ At various intervals after in-

jection the animals were etherized and bled from the subaxillary artery for the determination of the penicillin blood concentration by the method in current use.¹⁰ At corresponding intervals other groups were infected. One cc of an 18 hour serum-broth culture of *Streptococcus* strain 4 (Group A, Type 3) and *Pneumococcus* Type 1 strain 6301 was inoculated intraperitoneally into the rats previously treated with the penicillin preparation. The inocula varied from 10^{-5} to 10^{-7} for the pneumococcus and from 10^{-4} to 10^{-6} for the streptococcus, corresponding to 100 - 1000 LD. Groups of 10 to 20 rats were used for control and for each alteration in the interval, or dosage, in any single experiment. Most of the untreated control animals died within 2 to 3 days. The rats which survived a three-week period were adjudged cured; occasionally the effect of treatment was verified by heart cultures of the animals sacrificed at the end of the observation period.

Results. (a) *Blood levels.* In our initial attempts to establish the duration of the penicillin levels, we encountered a number of

* Part of this material was presented at the meeting of the American Societies of Experimental Biology, Atlantic City, May, 1948.

¹ Tillet, W. S., Cambier, M. J., and McCormack, J. E., *Bull. N. Y. Acad. Med.*, 1944, **20**, 142.

² Tillet, W. S., McCormack, J. E., and Cambier, M. J., *J. Clin. Invest.*, 1945, **24**, 589.

³ Zubrod, C. G., *Bull. Johns Hopkins Hosp.*, 1947, **81**, 400.

⁴ White, H. J., Baker, M. J., and Jackson, E. R., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 199.

⁵ Gibson, C. D., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 278.

⁶ Marshall, E. K., Jr., *Bull. Johns Hopkins Hosp.*, 1948, **82**, 403.

⁷ Eagle, H., *Ann. Int. Med.*, 1948, **28**, 260.

⁸ Schuchter, R. J., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 29.

TABLE I.
Duration of Detectable Penicillin Levels in Rats Treated with Penicillin in Oil + 1 mg/cc Epinephrine.

Injection: 0.1 cc/100 g, intramuscular.
Rats of 80-150 g weight.

Dose/100 g	Hr duration in	
	50% rats	90% rats
5 × 1000	13	14
10 × 1000	14	16
20 × 1000	16	18

⁹ Ercoli, N., Hueper, W. C., Landis, L., Schwartz, B. S., and Queally, F. J., *J.A.M.A.*, 1948, **138**, 115.

¹⁰ Rammelkamp, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 95.

TABLE II.
Protection of Rats Against *Pneumococcus* Type I Infection at Various Intervals After
Repository Penicillin Treatment.

Dose u/100 g†	Hr after treatment	Blood negative for hr	Survivors after			
			6 days		21 days	
			Survivors infected	% survivors	Survivors infected	% survivors
Controls	—	—	26/134	19.4	14/134	10.4
10,000	14	*	8/ 10	80	8/ 10	80
	17	1	11/ 20	55	6/ 20	30
	20	4	8/ 20	40	4/ 20	20
	22	6	7/ 10	70	6/ 10	60
	24	8	14/ 25	56	7/ 25	28
	28	12	2/ 10	20	2/ 10	20
	16	*	7/ 10	70	6/ 10	60
	18	†	20/ 30	66.6	16/ 30	53
	20	2	31/ 50	62	21/ 50	42
	25	7	6/ 13	46	2/ 13	15.4
20,000	35	17	4/ 13	31	1/ 13	7.7

* 50% of rats negative.

† 90% " " " "

‡ in 0.1 cc/100 g.

discrepancies. From experiments conducted on a large number of rats, it became apparent that the absolute weight of the animal—using the same dose/weight—influences the duration of blood levels. The larger animals (over 150 g) gave longer durations due to the greater absolute dose injected. In order to minimize variables, only experiments conducted on rats of 80-150 g weight are considered in this paper.

The duration of blood level is expressed in the most significant manner by indicating the end period of detectable penicillin in 50% and 90% of the animals. (Table I). The establishment of this effect in 100% of the animals is exposed to greater error, as is the case in other dose-response relationships.

(b) *Pneumococcus* Type I Infection. From the experiments presented in Table II, it is evident that the therapeutic activity outlasts the duration of the detectable blood level. For instance, rats infected 20 hours after treatment had no penicillin in their blood for the last 2 hours before infection, yet in 62% death was significantly delayed and 42% were cured. In general, a definite protective effect was given by the repository treatment 6-8 hours after the end of the penicillin blood level.

(c) *Hemolytic Streptococcus* Infection. Four experiments were carried out on a total

of 250 rats. The same bacterial strain was used for *in vitro* as for *in vivo* experiments. The results are not as clear-cut as in the pneumococcal infection, due probably to the heavier inocula used in order to compensate for the less regular susceptibility of rats to streptococcal infection. However, in this infection also, chemotherapeutic effectiveness appears to be present for relatively long periods (4-8 hours) following non-detectable blood levels. In one experiment, for instance, out of 30 rats infected 2 to 6 hours after the disappearance of penicillin from the blood, 10 rats survived an observation period of 21 days. Seventy-five percent of a group of 20 controls died within 2 days; the entire group within 8 days.

Conclusion. It may be concluded that our present bacteriological methods cannot give the value of the *minimal* penicillin concentrations required for therapeutic activity. It is certain that this concentration in the heavy bacterial infection used is below 0.015-0.03 u/cc serum, which is the sensitivity of the bacteriological method.

Our present finding explains only in part the well-documented opinion of other authors that it is not necessary to maintain continuous penicillin blood levels for the whole period of treatment. A more important factor responsible for this is probably the *additive chemo-*

therapeutic effect of repeated doses. This effect is obvious from the experiments reported in the literature indicating that *fractions* of the curative dose, repeated at such intervals as to leave the organism without penicillin for the greatest part of the treatment period, result in cure. This is the case in spirochetal¹¹ as well as in bacterial³ infections. The *additive effect* might depend on biological changes induced by sub-curative doses on the micro-organism, on reduction of the bacterial population, and on the immunological response of the host. The experiments of Kelly and Schnitzer¹² would suggest furthermore that the immunological response might influence the susceptibility of the micro-organism to chemotherapy.

The immunological response itself can be influenced by the treatment schedule, according to the recent observations of Kilbourne and Loge.¹³ In hemolytic streptococcal pharyngitis, the antistreptolysin titer is

¹¹ Eagle, H., Magnuson, H. J., and Fleischman, R., *Bull. Johns Hopkins Hosp.*, 1946, **79**, 168.

¹² Kelly, D. R., and Schnitzer, R. J., *Arch. Biochem.*, 1945, **7**, 461.

higher with *intermittent* penicillin injections than it is with continuously sustained blood levels.

Another phenomenon which accounts for the independence of therapeutic effect duration from blood level is revealed in the experiments of Grunberg, Schnitzer, and Unger.¹⁴ Penicillinase injected *after* the total disappearance of penicillin from the host tissue still blocks the therapeutic effect in the local streptococcal infection of mice. Thus, the presence of the penicillin in the micro-organism is more prolonged than in the host.

In our own experiments, part of the therapeutic effect existing beyond the blood level is due to penicillin accumulated in the tissues. How much of this prolonged therapeutic effect is dependent upon the undetectable amounts present in the blood, in comparison with the penicillin accumulated in the organs and tissue fluids, remains an open question.

¹³ Kilbourne, E. D., and Loge, J. P., *J. Clin. Invest.*, 1948, **27**, 418.

¹⁴ Grunberg, E., Schnitzer, R. J., and Unger, C., *Yale J. Biol. and Med.*, 1948, **20**, 479.

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4-Amino Pteroylglutamic Acid (Aminopterin), Pteroylglutamic (Folic) Acid, and Response of Frogs, *Rana clamitans*, to Estrogens.*

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It has been shown that chicks and monkeys (*Macacus rhesus*) maintained on a diet deficient in pteroylglutamic (folic) acid exhibit a decreased response to estrogenic substances.¹⁻³ It has been further demonstrated

by Franklin, Stokstad, and Jukes⁴ that folic acid deficiency in mice may be accelerated by a chemical antagonist of folic acid ("crude antagonist", Lederle). Hertz,⁵ using the same antagonist, has shown that it interferes with the estrogen response of the chick genital

* This investigation was supported by a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

¹ Hertz, R., *Endocrinology*, 1945, **37**, 1.

² Hertz, R., *Recent Progress in Hormone Research*, II, Academic Press, 1948, 161.

³ Hertz, R., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 113.

⁴ Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 368.

⁵ Hertz, R., *Science*, 1948, **107**, 300.

TABLE I.
Effects of Various Levels of 4-Aminopteroylglutamic Acid, Pteroylglutamic Acid, and Estradiol Benzoate on the Growth of Oviducts of *Rana clamitans*.

Series No.	No. of frogs	Aminopterin per inj., mg	Folic acid per inj., mg	No. of inj. per wk	Estradiol per wk, mg	Oviduct response of female frogs	
						After 2 wks of estradiol	After 3 wks of estradiol
I*	9	.05	0	3	.1	+	+
"	10	.10	0	3	.1	+	+
"	5	0	2.0	3	.1	+++	++++
"	5	.10	1.0	3	.1	+	+
"	10†	0	0	0	.1	+++	++++
"	5	0	0	0	0	—	—
II*	5	.05	0	3	.1	+	+
"	13	.10	0	3	.1	+	+
"	11	.20	0	3	.1	+	+
"	2	1.0	0	2	.1	+	+
"	5	0	1.0	3	.1	++++	++++
"	5	0	2.0	3	.1	++++	++++
"	5	.05	1.0‡	3	.1	+	+
"	4	.1	2.5‡	3	.1	+	+
"	5	.25	2.5‡	3	.1	+	+
"	5	0	0	0	0	—	—
"	5	0	0	0	.1	+++	+++

* Series I animals were simultaneously treated with estradiol and with substance(s) listed. Series II animals were pretreated for 2-3 weeks with the drug(s) listed and then in addition were injected with estradiol. (Administration of the drug(s) aminopterin, folic acid, or both were continued throughout estradiol treatment.)

† 5 animals of this group also received 0.4 cc of distilled water 3 times per week.

‡ Folic acid level was raised after 2 weeks treatment to 5 mg per injection.

+ Slight oviduct enlargement, no coiling.

+++ Oviducts enlarged and coiled.

++++ Marked oviduct enlargement and coiling.

— Oviducts small, thin, and uncoiled.

tract. The 4-amino analogue of pteroylglutamic acid was described as being strongly antagonistic to folic acid in the *Streptococcus fecalis* R test.⁶ Goldsmith, Tobias, and Harnly⁷ reported that both the "crude antagonist" and 4-amino pteroylglutamic acid (aminopterin) prevented the development of *Drosophila melanogaster* larvae into adults. This inhibition by the "crude antagonist" could be overcome in a number of cases by the addition of folic acid to the diet. However, thus far, the inhibitory effect of aminopterin on the development of *Drosophila* larvae has not been counteracted or reversed by several levels of folic acid. Franklin, Stokstad, and Jukes⁸ found that mice died within a few days after being placed on a diet containing 1 mg or more of aminopterin per kilo of diet.

At a level of 0.3 mg per kilo the aminopterin depressed the blood values. This could be reversed by high dosages of folic acid. However, large amounts of folic acid did not overcome the effect resulting from higher concentrations of aminopterin. Recently, Hertz described the decreased response of the chick⁹ and the rat¹⁰ to estrogen following treatment with aminopterin. High levels of folic acid reversed this effect.¹⁰

In the light of the above data, and since it had been established previously that estradiol injections in newly metamorphosed frogs are followed by a pronounced oviduct response within 2 to 3 weeks,¹¹ it was considered worth-

⁸ Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 398.

⁹ Hertz, R., *Proc. 39th Annual Meeting Am. Assn. Cancer Research*, March 1948.

¹⁰ Hertz, R., *personal communication*.

¹¹ Schreiber, S., and Rugh, R., *J. Exp. Zool.*, 1945, **99**, 93.

⁶ Seeger, D. R., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1947, **69**, 2567.

⁷ Goldsmith, E. D., Tobias, E. B., and Harnly, M. H., *Anat. Rec.*, 1948, **101**, 104.

while to ascertain the action of aminopterin and its effect on the estrogen response in frogs.

Experimental. This paper presents preliminary data on a group of about 100 newly metamorphosed frogs (*Rana clamitans*) treated parenterally with various dosage levels of aminopterin, folic acid, and estradiol benzoate. The course of treatment and levels of drugs administered are outlined in Table I. The frogs were divided into two series. In series I, the frogs were treated with aminopterin[†] or folic acid,[‡] or both, and were simultaneously treated with estradiol benzoate.[‡] In series II, frogs were pretreated with aminopterin alone, folic acid alone, or both for a period of 2 to 3 weeks. At this time, estradiol was added to this regimen. The volume of fluid injected was controlled as closely as possible, and no frog received more than 0.4 cc of fluid per injection. In addition, the site of puncture was carefully held closed to prevent any leakage of injected fluid.

The data indicate that treatment with aminopterin decreased the response of the female frog oviducts to estradiol. Oviducts of the estradiol treated females showed marked enlargement and coiling. However, the oviducts of the females which received estradiol and aminopterin simultaneously, or which were pretreated with aminopterin and then were given estradiol plus aminopterin,

showed very slight enlargement and no coiling.

Simultaneous administration of folic acid and estradiol was followed by oviduct growth and coiling in the females equal to, but not greater than that observed in the females treated with estradiol alone. However, pretreatment with folic acid for 2 to 3 weeks followed by injections of estradiol plus folic acid resulted in oviduct growth which was greater than that observed in the females which received only estradiol.

In 4 groups of frogs, in addition to aminopterin and estradiol, supplements of folic acid were administered in ratios of 10, 50 and 100 to 1 of folic acid to aminopterin. In none of these female frogs were the oviducts grossly different from those in the females treated only with aminopterin and estradiol.

The data also indicate that those animals treated with aminopterin appeared to show a higher mortality rate than those receiving supplements of folic acid in addition to aminopterin. The majority of aminopterin treated frogs which died, did so 3 to 4 weeks following initiation of treatment with aminopterin, irrespective of level of drug administered.

Histological studies of the oviducts and other organs such as liver, spleen, and adrenal will be reported at a later date.

Summary. 4-amino pteroylglutamic acid (aminopterin) markedly decreased, whereas, folic acid increased the response of the oviducts of newly metamorphosed female frogs (*Rana clamitans*) to estradiol. The aminopterin effect could not be reversed by folic acid in ratios as high as 100:1 of folic acid to aminopterin.

[†] The 4-amino pteroylglutamic acid was generously supplied by the late Dr. Y. Subbarow, and the pteroylglutamic acid by Dr. A. L. Franklin of Lederle Laboratories.

[‡] The estradiol benzoate was generously supplied by Dr. F. F. Yonkman and Dr. F. L. Mohr of Ciba Pharmaceutical Products.

A New Method for the Electrical Recording of Mechanical Deformations.

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Pressure changes or spacial displacements may be recorded by a number of electrical devices,¹⁻¹⁰ based on changes in capacity, resistance or the piezo electric effects. Variations in potential are amplified and recorded photographically. On referring to these methods Green¹¹ states that "while many of them are admirable in concept, their complexity and cost have so far prevented their wide acceptance".

The system about to be described is of low cost, easy to make and of almost universal application. It depends on the variations in resistance occurring in a glass capillary (c) containing equal volumes of alcohol and glycerine; copper wires are inserted into the two ends one of which is fixed (e_2) and the other (e_1) which is movable, being connected to a membrane (m) or any other mechanical device which allows for displacement in space. The fixed electrode (e_2) can be moved in relation to the movable one (e_1) by means

of a screw. The movable electrode should be of small diameter (approximately 0.75 mm) so its mass will have no influence on the experiment. The ends of the electrodes are connected to points a and b of the electric system represented in Fig. 2, consisting of a battery of approximately 10 volts and a switch I_1 , which on closing sends the current through the variable resistance R_1 . The proper voltage is obtained by altering the position of the cursor in this resistance. The

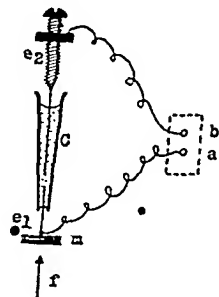


FIG. 1.

A fixed electrode (e_2) and a movable one (e_1) are inserted into the capillary tube C, which contains the electrolyte (alcohol and glycerine). The electrode e_1 is joined to the membrane (m), which is displaced by the force (f). Terminals a and b are connected to points similar to those shown in Fig. 2.

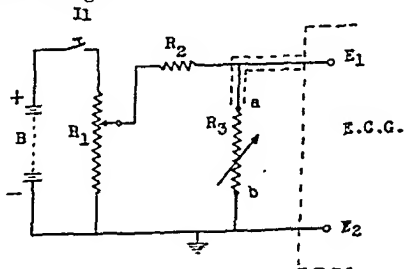
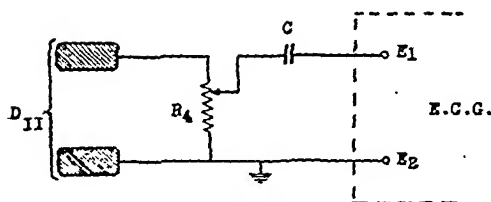


FIG. 2.

The electrical circuit consists of a battery B, a switch I_1 , and resistances R_1 and R_2 , each 250,000 ohms. Variable resistance R_3 corresponds to the capillary system in Fig. 1. The union between a and E_1 is shielded. E_1 and E_2 are entrances to the electrocardiograph (E.C.G.).

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- 3 Hampel, A., *Pflüg. Arch. ges. Physiol.*, 1940, **244**, 141.
- 4 Rein, H., Hampel, A., and Heinemann, W., *Pflüg. Arch. ges. Physiol.*, 1940, **243**, 329.
- 5 Macleod, A. C., and Cohn, A. E., *Am. Heart J.*, 1941, **21**, 345.
- 6 Miller, A., and White, P. D., *Am. Heart J.*, 1941, **21**, 504.
- 7 Dalla Torre, L., *Helv. Physiol. Acta.*, 1943, **1**, C14.
- 8 Grundfest, H., Hay, J. J., and Feitelberg, S., *Science*, 1945, **101**, 255.
- 9 Lambert, E. A., and Wood, E. H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 186.
- 10 Motley, H. L., and Courmand, A., *et col.*, *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 241.
- 11 Green, H. D., *Circulation, Physical Principles*, in O. Glasser, *Medical Physics*. The Year Book Publ., Inc., Chicago, 1944, p. 227.



$D_{II} = \text{lead 2.}$

FIG. 3.

Additional circuit for recording of R wave of E.C.G. $D_{II} = \text{lead 2.}$ $R_4 = 250,000$ ohm variable resistance. $C = 0.2 \mu F$ condenser.

potential differences produced at the extremes of R_3 (the capillary's electrolytic resistance) may be amplified through a clinical electrocardiograph (E.C.G.) or another amplifying device, when the movable electrode is displaced.

It has been possible to make an immediate and accurate record of a whole range of mechanical manifestations in the circulation system by means of this apparatus. Suitable capsules are used in each case as fully described elsewhere.

In order to relate the mechanical activities recorded in this way with other manifestations of the heart's activity we have superimposed on the mechanical tracing the R wave of the electrocardiogram by using an additional circuit with a variable resistance and a condenser (Fig. 3).

The different tracings obtained in man are recorded in Fig. 4: carotid pulse (I), jugular pulse (II), venous pulse together with the R wave of the electrocardiogram (III), radial pulse and R wave (IV), and finally the apex beat together with the R wave of the electrocardiogram (V).

Further applications of this method will be described later.

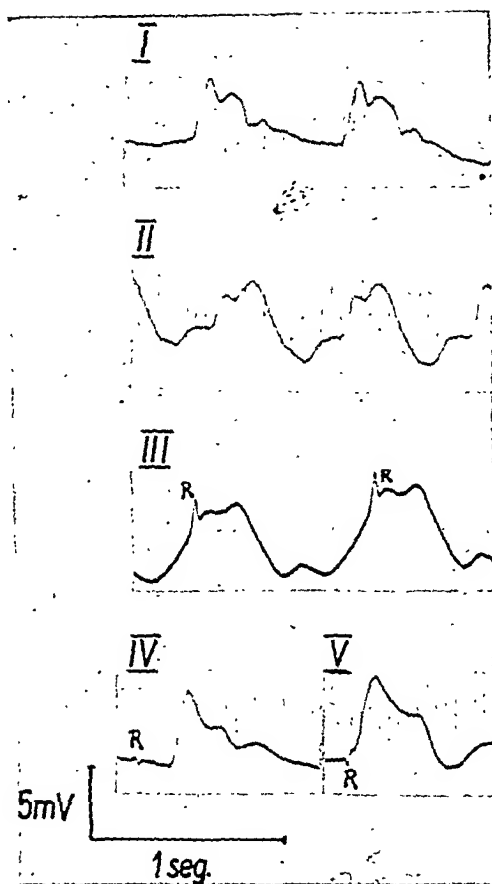


FIG. 4.

Tracings obtained in men: (I) Carotid pulse. (II) Venous pulse (jugular vein). (III) Venous pulse and R wave of the E.C.G. (IV) Radial pulse and R wave. (V) Apex-beat and R wave.

Summary. A new electrical method for recording mechanical variations is described which uses the differences in potential obtained by changes in the electrolytic resistance of a glass capillary filled with alcohol and glycerine. The method is specially useful in the study of mechanical changes in the cardio-vascular system.

¹² Günther, B., and Coneha, J., *Rev. Argent. Cardiol.*, 1948, in press.

Influence of Vaccine Virus on a Transmissible Leukemia of Mice.*

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The relation of extraneous viruses to neoplasms has been the subject of comparatively few investigations, scattered over the past 25 years. Levaditi and his associates^{1,2} found that certain pox viruses proliferate in epitheliomas of rodents and sometimes cause necrosis and impairment of growth of the tumors. Rivers and Pearce^{3,4} studied the influence of Virus III on a rabbit carcinoma, but could find no appreciable alteration of the degree of malignancy.

More recently, it has been demonstrated that vaccine virus will proliferate in mouse sarcoma 180, and furthermore, that transplants of fragments of infected tumor grow much more slowly and less successfully than controls.⁵ The explanation was offered that this effect was produced by a direct action of the virus on the neoplastic cells, perhaps by a kind of competitive biochemical antagonism. The study has now been extended to leukemias of mice. These neoplasms provide an exceptionally satisfactory test material, both in their close resemblance to the corresponding human disease and in their readily predictable and high grade of malignancy.

Mice of a closely inbred line of the Ak strain and a leukemia designated 9417 have

been employed. This tumor is lymphoid in type and at post-mortem the dominant lesions are usually found to be in liver, spleen, and mesenteric nodes, all of which may be greatly enlarged.

The strain of vaccine virus used was adapted to mouse brain more than a year ago, and has been carried along since by frequent intracerebral passage.⁵ It is lethal when inoculated directly into the brain, but moderate doses may be given subcutaneously or intravenously without the development of any gross lesions or impairment of health. The virus was given as a suspension of infected mouse brain in 5 parts of plain broth containing 1,000 units per cc of both penicillin and streptomycin and 0.5 mgm heparin.

Leukemia 9417 is readily transmitted within this strain of mice, becoming established in, and killing approximately 100% of the hosts within a few weeks.⁶ For transmission, the enlarged spleen of an animal dying of the disease was macerated in 10 cc of normal saline, and 0.1 cc was injected intraperitoneally.

In a first experiment leukemic cells were implanted in 50 young mice. Of these, 23 were kept as controls and received no further treatment. The remaining 27 were given vaccinia virus parenterally after periods of time sufficiently long to allow no reasonable doubt that the leukemia had meanwhile become well-established. One group of 17 received on the seventh day after the leukemic transplant 0.2 cc of vaccine virus suspension intravenously. A second group of 10 mice were given, on the fifteenth day, 0.2 cc of virus suspension intravenously and at the same time 0.5 cc intraperitoneally. The rates at which the control and the treated

* This investigation was aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the Holmes Foundation, Inc., and the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

¹ Levaditi, C., and Nicolau, S., *Compt. rend. Soc. de biol.*, 1922, **87**, 498.

² Levaditi, C., and Nicolau, S., *Compt. rend. Acad. de Sc.*, 1922, **174**, 1649.

³ Pearce, L., and Rivers, T. M., *J. Exp. Med.*, 1927, **46**, 81.

⁴ Rivers, T. M., and Pearce, L., *J. Exp. Med.*, 1925, **42**, 523.

⁵ Turner, J. C., and Mulliken, B., *Cancer Research*, 1947, **7**, 774.

⁶ Burchenal, J. H., Lester, R. A., Riley, J. B., and Rhoads, C. P., *Cancer*, 1948, **1**, 399.

TABLE I.
Number of Mice Surviving After Inoculation of Leukemic Cells.

Day:	13	16	19	22	25	54
Controls	23/23	3/23	0	—	—	—
Treated with virus on Day 7	17/17	7/17	6/17	4/17	2/17	2/17
Treated with virus on Day 15	10/10	7/10	6/10	4/10	2/10	2/10

TABLE II.
Number of Mice Surviving After Inoculation of Leukemic Cells.

Day:	13	16	19	22	25	28	35
Controls	19/20	16/20	9/20	4/20	2/20	1/20	1/20
Treated with virus on Day 7	13/15	7/15	5/15	4/15	4/15	4/15	4/15
Treated with virus on Day 12	15/15	12/15	7/15	4/15	2/15	2/15	2/15

animals died may be seen in Table I.

It may be noted that all of the control mice were dead on the 19th day after inoculation of leukemic cells. In contrast, deaths occurred less rapidly in both groups receiving virus, and no less than 4 animals (15%) were still alive at least until the 54th day after transplantation of leukemia. Two have survived 194 days.

The experiment was repeated with the modification that the group of animals treated late in the disease received the injection of virus on the 12th day instead of on the 15th day. There were 20 control mice and 15 mice in each of the groups receiving virus. Table II records the findings.

It is apparent that the leukemia is now somewhat less acute than before, yet among the controls the mortality at the end of the 4th week is nearly 100%. A difference between the groups is first evident at that time when, with all but one of the control animals dead there are still 6 (20%) of the 30 treated mice alive. Furthermore, 4 of these have now survived at least until the 170th day after transplantation of the leukemia.

All of the mice that died, both treated and untreated, showed gross evidence of leukemia, with enlargement of liver, spleen, or lymph nodes. Portions of the organs removed from animals that had received virus were examined for vaccinia by intradermal injection of rabbits and by inoculation of chorio-allantoic membranes. Virus was demonstrable in about 25%. Whether this represents a fair estimate of the presence of virus is not clear, since it may well be that antibody

was circulating in sufficient concentration to interfere with the tests.

It would appear that the introduction of vaccinia virus by parenteral injection in the course of mouse leukemia may result in the prolongation of life of a significant number of hosts. Whether the animals still alive will ultimately die of leukemia remains to be seen. It could be supposed that they will, and that the delay in the time of their death is merely a consequence of undernutrition brought on by systemic vaccinal infection; but no evidence of appreciable complicating general injury to the hosts has yet been uncovered. The weights of all groups of animals were taken several times a week, and none failed to gain.

It is hardly possible to compare the prolongation of life of leukemic mice brought about by orthodox chemotherapeutic or physical agents with the effect observed as a result of the injection of virus. In one case it is customary to employ repeated doses of the material on trial and to begin very soon after the leukemia has been transplanted into the subject. Here, on the other hand, a dose of virus has been given only on a single day and that only relatively late in the course of the disease.

The mechanism of the observed effect is not clear. It is reasonable to suppose that a direct action of virus on neoplastic cells takes place, but the reaction may then be modified by the operation of immune bodies.

Summary. In 2 experiments, 100 mice of the inbred Ak strain were inoculated with leukemia 9417. One week or two weeks later

57 animals received parenteral injections of living vaccinia virus. The 43 controls received no treatment.

Of the controls all but one (98%) were dead within 4 weeks after the leukemic transplant. The lives of at least 10 (18%) of the mice receiving virus seemed to be sig-

nificantly prolonged, and 6 of these have survived more than 5 months.

The authors wish to express their appreciation for the generosity of Dr. Joseph Burehenal of the Sloan-Kettering Institute, who supplied the tumors, as well as most of the mice employed.

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Search for the Brown-Pearce Tumor XYZ Factor in Rabbit Spleen.

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Some years ago it was reported that frozen and preserved Brown-Pearce tumor tissue contained a factor called, for lack of a better term, the XYZ factor, which, when injected 2 weeks before the Brown-Pearce tumor transplantation resulted in increased incidence of, and larger metastases, and a shorter survival period after transplantation.¹⁻⁴ The factor was different from the Duran-Reynals spreading factor (hyaluronidase),⁵⁻⁸ nor was the factor present in normal rabbit testicle,^{5,6} in the tissue of an adenocarcinoma of the rabbit uterus (Greene),⁹ in Bashford Carcinoma 63,¹⁰ nor in Sarcoma 180 of the mouse.¹⁰ A review of the literature revealed

that Haaland¹¹ and Leitch¹² had described a similar factor for Bashford Carcinoma 63 and it was possible to confirm their work.¹³ The Bashford Carcinoma 63 XYZ factor seemed to be specific in that the growth of this tumor was not affected by similarly prepared material from mouse Carcinoma 48, mouse Sarcoma 37, nor by the XYZ factor from the Brown-Pearce tumor.^{14,15} Much has been written concerning the presence of inhibiting and enhancing materials said to be present in the spleen.¹⁶ In resuming work on the Brown-Pearce XYZ factor after a lapse of some 10 years, preliminary experiments were performed in an attempt to recover the factor from the spleens of normal rabbits and of rabbits 2 weeks after the injection of the factor.

Materials and methods. In the 2 experiments 52 rabbits were employed. Each rabbit was about 3 months of age, except for

* The work was aided by a grant from the American Cancer Society, through the Committee on Growth of the National Research Council.

1 Casey, Albert E., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 816.

2 Casey, Albert E., *Am. J. Cancer*, 1934, **21**, 760.

3 Casey, Albert E., *Am. J. Cancer*, 1934, **21**, 776.

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7 Coman, Dale R., McCutcheon, Morton, and Ziedman, Irving, *Cancer Research*, 1947, **7**, 383.

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13 Casey, Albert E., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 674.

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15 Casey, Albert E., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 663.

16 Stern, K., and Willheim, R., *The Biochemistry of Malignant Tumors*, Reference Press, Brooklyn, N. Y., 1943, 714.

TABLE I.
Course of Brown-Pearce Tumor in Rabbits Transplanted Subcutaneously.

	Rabbits, No.	Tumor takes	Tumor per animal inoculated			Per animal with tumor
			Met. foci, No.	Vol. met., cc	Total tumor, cc	Met. foci, No.
Spleen (XYZ)	12	3 (25%)	0.3	2.1	11.7	1.3
Spleen (normal)	12	4 (33%)	1.8	10.7	18.5	5.5
Controls	13	3 (23%)	1.7	19.3	33.4	7.3
XYZ (BP tumor)	3	2 (67%)	10.7	45.0	112.7	16.0
8 Prior Experiments with the Subcutaneous Inoculation of BP Tumor.						
Controls	52	16 (31%)	2.7	12.8	21.6	4.4
XYZ (BP tumor)	58	44 (76%)	7.7	31.1	46.7	10.2

7 adult males used in obtaining the transfer material and in the preparation of the spleens. The young animals were divided into matched groups of the same breed and all were obtained from the same local breeder.

The spleens were prepared as follows: Two normal rabbits were killed by air injection and the spleen removed aseptically, placed in a sterile tube and stored in the deep freeze chamber at 0°F. The preserved spleen after 14 days in the first and 68 days in the second experiment, was removed from the deep freeze, emulsified in normal saline (1-3 dilution) and 0.3 cc of the emulsion injected subcutaneously into 5 rabbits in the first experiment and into 8 rabbits in the second experiment.

For XYZ material a rabbit bearing the Brown-Pearce tumor was killed by air injection 13 days after intratesticular transplantation. The large testicular tumor was removed aseptically, 1.5 cc used immediately for transfer (A) and 6 cc placed into sterile tubes without glycerin or other preservative and stored at 0°F (B). The transfer tissue (A) was emulsified in 4 cc of normal saline and 0.2 cc of the emulsion injected subcutaneously or intratesticularly into 15 rabbits. The transplantation was successful indicating that the tumor used was viable. One portion of the frozen tumor tissue (B) was removed 7 days later, 3 cc emulsified in 10 cc of normal saline and 0.33 cc of the emulsion was injected subcutaneously into the back of 2 rabbits which were killed by air injection 16 days later, the spleens removed aseptically, placed in sterile tubes, and stored in the deep freeze chamber at

0°F (C). The spleens thus obtained (C) were removed from the deep freeze after 14 days in the first and 68 days in the second experiment, emulsified in normal saline (1-3 dilution) and 0.3 cc of the emulsion injected subcutaneously into 5 rabbits in the first and into 8 rabbits in the second experiment. In the first experiment a second batch of frozen Brown-Pearce tumor tissue (XYZ material, marked B) was removed after 37 days in the deep freeze, emulsified in normal saline (1-3 dilution) and 0.3 cc injected subcutaneously into 5 rabbits. The material used was sterile when cultured in thioglycollate medium, and microscopic sections of biopsies of the frozen and fresh spleen and tumor were made and examined at each stage of the experiment.

In the first experiment, 8 days after injection of the spleen emulsions and the Brown-Pearce XYZ material, each animal in the 3 groups (with spleen from XYZ animal, with spleen from normal animal, with Brown-Pearce XYZ material; one animal had died meanwhile of intercurrent disease) together with the previously matched but uninoculated controls were injected subcutaneously into the back in numerical order with an emulsion of Brown-Pearce tumor tissue in normal saline.

In the second experiment there were 3 groups of 8 animals in each. The first group was injected with an emulsion of spleen preserved 68 days at 0°F from the animal which had received Brown-Pearce XYZ (as described above); the second group was injected with an emulsion of spleen taken from a

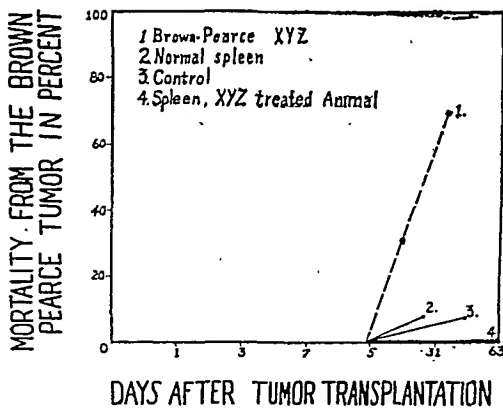


FIG. 1.

normal rabbit and preserved for 68 days at 0°F; the third group were control animals. Twenty days after the injection of the splenic emulsions into the 2 experimental groups the animals of these 2 and those of the control group (24 animals in all) were injected subcutaneously with 0.2 cc of an emulsion (1-3 dilution) of Brown-Pearce tumor tissue in numerical order. In addition a stock animal (NZW) was injected intratesticularly with 0.2 cc of the emulsion. Biopsy showed that this tumor was nearly all living, the animal having been inoculated intratesticularly.

Results. The results are summarized in Fig. 1 and in Table I. One animal from the controls and one from the spleen XYZ series, another from the Brown-Pearce XYZ group and one from the normal spleen group died of intercurrent disease between 2 and 17 days after tumor transplantation. These were eliminated from the results because mortality from the tumor does not occur until 20 days or longer after inoculation.

There was no statistically significant difference in the success of transplantation, in the mortality, in the number of metastatic foci, nor in their volume, nor in the total tumor primary and metastatic per animal inoculated between the 3 groups. There was no evidence that the emulsions of the splenic tissue had in any way significantly affected the course of the Brown-Pearce tumor.

The Brown-Pearce XYZ group containing only 3 animals could not be analyzed statistically but it was consistent with previous

experience on the intracutaneous inoculation of the Brown-Pearce tumor (Table I). In 8 prior experiments at the Rockefeller Institute,³ at the Louisiana State University¹⁰ and at the Baptist Hospitals¹⁰ there were 52 controls and 58 B.P. XYZ animals inoculated with Brown-Pearce tumor subcutaneously into young rabbits. The incidence of tumor proven by autopsy was among all animals inoculated, 31% in the controls and 76% among the XYZ treated animals, metastatic foci 2.7 and 7.7, volumes of metastatic tumor 12.8 and 31.1 cc, total tumor 21.6 and 46.7 cc; metastatic foci among animals with tumor was 4.4 in the controls and 10.2 in the XYZ animals. The results in the XYZ group in the present experiments were in accord with this prior data.

Discussion. Stern and Willheim published a review of the voluminous literature on the effect of spleen extracts on transplanted tumors.¹⁶ Much of the work reviewed was done without controls or without an adequate number of animals for statistical analysis. Woglom¹⁷ found that fresh splenic implants increased resistance to tumor transplantation but that crushed, frozen or thawed mouse spleen had no effect upon resistance to Bashford carcinoma 63 of the mouse. Fardon, Brotzge and Loeffler¹⁸ obtained resistance to mouse mammary carcinoma 15091 A by means of extracts of fresh (unfrozen) mouse spleen and not with heterologous spleen. Since our work was done with frozen and not with fresh spleen it is in accord with previously published work and there is no reason to suspect that the Brown-Pearce XYZ factor may be found in the spleen of normal or of XYZ injected animals.

Summary. The Brown-Pearce tumor XYZ factor could not be prepared from the spleen of normal rabbits nor from the spleens of rabbits previously injected with the factor. There were two experiments comprising 52 rabbits and further controlled by prior experiments involving 110 young rabbits likewise injected intra- or subcutaneously with the Brown-Pearce tumor.

¹⁷ Woglom, W. H., *J. Exp. Med.*, 1910, 12, 29.

¹⁸ Fardon, J. C., Brotzge, G. C., and Loeffler, M. K., *Studies of Inst. Divi Thomae*, 1911, 3, 69.

Influence of Alloxan Diabetes on Coagulability of the Blood.*

MARIO STEFANINI† (Introduced by Armand J. Quick.)

From the Department of Biochemistry, Marquette University, School of Medicine, Milwaukee.

The possibility that the pancreas may influence the coagulation of the blood has been considered by several investigators. Boldyreff^{1,2} has reported that pancreatectomy results in a temporary delay of the clotting time and that the establishment of a pancreatic fistula causes a significant and permanent prolongation of the clotting time as well as a bleeding tendency. Recently Hecht³ suggested a relationship between the internal secretion of the pancreas and the coagulability of the blood. He observed that diabetes was more common in families in which cases of sporadic hemophilia occurred, the criterion of sporadic hemophilia being the absence of the disease in the 4 preceding generations. This observation led Hecht to study the effect of alloxan on the clotting time in rats. He found that the clotting time of recalcified plasma was delayed in those animals which responded to alloxan and developed hyperglycemia.

In the present paper the influence of alloxan on the coagulation process was studied by means of the coagulation time and by various other methods which are more specific in detecting the basic disturbances in the coagulation mechanism.

Experimental. The studies were carried out on 10 adult male rabbits. The alloxan monohydrate in a 4% neutral solution in physiological saline was injected intravenously. The dose was of 200 mg per kilo of

body weight. To counteract the transitory hypoglycemia resulting from the drug, 5 cc of 50% glucose in water were given every 2 hours by stomach tube for the first 10 hours.

The blood sugar level was determined every 2 hours for the first 10 hours and then every 2 days using the method of Folin and Wu⁴ on venous blood obtained from an ear vein. On the second, fourth, seventh, tenth and fourteenth day samples of blood were obtained from the ear artery of the animal in a syringe coated with Silicone (General Electric Dri Film 9987) through a needle also coated with Silicone. Care was taken to avoid foaming and contamination with tissue juice. Two 1 cc samples of blood were immediately transferred to serological tubes with a uniform internal diameter of 11 mm, kept in a water bath at the constant temperature of 37°C and the clotting time determined according to a modified Lee-White technic.⁵ The remaining blood was decalcified by the addition of 0.1 M sodium oxalate in the ratio of one to ten. The prothrombin consumption was determined in the serum obtained from the samples of blood used for the determination of the clotting time according to the technic of Quick.⁶ The oxalated plasma, obtained by centrifuging the oxalated blood for 10 minutes at 2,000 r.p.m. was used for the determination of the clotting time of recalcified plasma, prothrombin time, relative concentration of the "labile factor", antithromboplastin and antithrombin activities. Both prothrombin time and clotting time of recalcified plasma were determined by the method of Quick.⁷ The

* This research was supported by a grant from the Division of Research Grants, National Institute of Health.

† Department of Internal Medicine, University of Roma, Italy. Senior Research Fellow, National Institute of Health.

¹ Boldyreff, W. N., *The Tohoku J. Exp. Med.*, 1937, **31**, 469.

² Boldyreff, W. N., *Erg. d. inn. Med. u. Kinderheilk.*, 1940, **59**, 29.

³ Hecht, E., *Acta Med. Scand.*, 1947, **129**, 311.

⁴ Folin, O., *J. Biol. Chem.*, 1929, **82**, 83.

⁵ Quick, A. J., Honorato, C. R., and Stefanini, M., *Blood*, 1948, **3**, 1120.

⁶ Quick, A. J., *Am. J. Med. Sc.*, 1947, **214**, 272.

⁷ Quick, A. J., *The Hemorrhagic Diseases and the Physiology of Hemostasis*, Charles Thomas, Springfield, Ill., 1942.

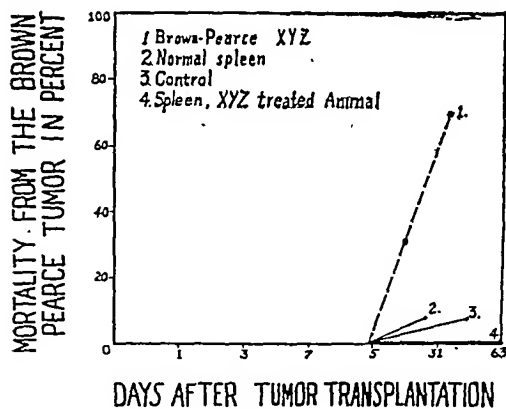


FIG. 1.

normal rabbit and preserved for 68 days at 0°F; the third group were control animals. Twenty days after the injection of the splenic emulsions into the 2 experimental groups the animals of these 2 and those of the control group (24 animals in all) were injected subcutaneously with 0.2 cc of an emulsion (1-3 dilution) of Brown-Pearce tumor tissue in numerical order. In addition a stock animal (NZW) was injected intratesticularly with 0.2 cc of the emulsion. Biopsy showed that this tumor was nearly all living, the animal having been inoculated intratesticularly.

Results. The results are summarized in Fig. 1 and in Table I. One animal from the controls and one from the spleen XYZ series, another from the Brown-Pearce XYZ group and one from the normal spleen group died of intercurrent disease between 2 and 17 days after tumor transplantation. These were eliminated from the results because mortality from the tumor does not occur until 20 days or longer after inoculation.

There was no statistically significant difference in the success of transplantation, in the mortality, in the number of metastatic foci, nor in their volume, nor in the total tumor primary and metastatic per animal inoculated between the 3 groups. There was no evidence that the emulsions of the splenic tissue had in any way significantly affected the course of the Brown-Pearce tumor.

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Discussion. Stern and Willheim published a review of the voluminous literature on the effect of spleen extracts on transplanted tumors.¹⁶ Much of the work reviewed was done without controls or without an adequate number of animals for statistical analysis. Woglom¹⁷ found that fresh splenic implants increased resistance to tumor transplantation but that crushed, frozen or thawed mouse spleen had no effect upon resistance to Bashford carcinoma 63 of the mouse. Fardon, Brotzge and Loeffler¹⁸ obtained resistance to mouse mammary carcinoma 15091 A by means of extracts of fresh (unfrozen) mouse spleen and not with heterologous spleen. Since our work was done with frozen and not with fresh spleen it is in accord with previously published work and there is no reason to suspect that the Brown-Pearce XYZ factor may be found in the spleen of normal or of XYZ injected animals.

Summary. The Brown-Pearce tumor XYZ factor could not be prepared from the spleen of normal rabbits nor from the spleens of rabbits previously injected with the factor. There were two experiments comprising 52 rabbits and further controlled by prior experiments involving 110 young rabbits likewise injected intra- or subcutaneously with the Brown-Pearce tumor.

¹⁷ Woglom, W. H., *J. Exp. Med.*, 1910, **12**, 29.

¹⁸ Fardon, J. C., Brotzge, G. C., and Loeffler, M. K., *Studies of Inst. Divi Thomae*, 1911, **3**, 69.

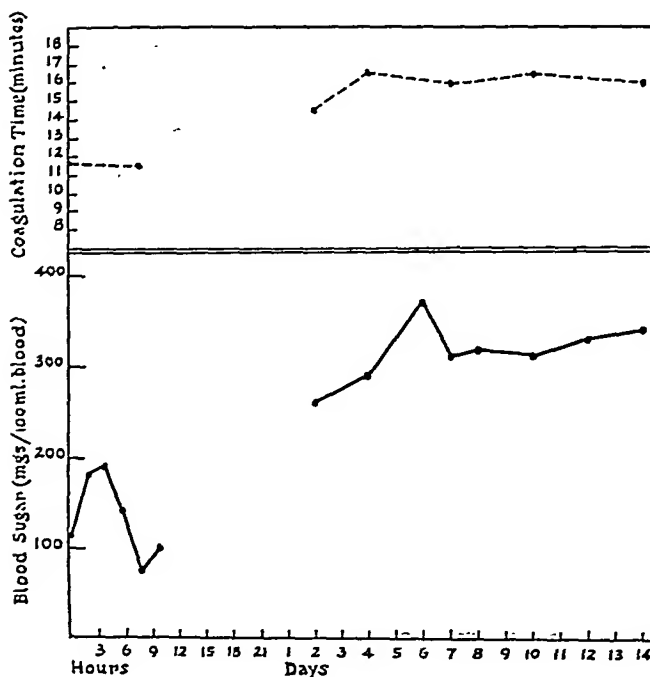


FIG. 1.

Behavior of the clotting time of whole blood and blood sugar level after the intravenous injection of alloxan monohydrate (200 mg per kilo body weight) in rabbits (average figures).

strength prepared by serial dilution in physiological saline. Normal and pathological plasmas also showed a similar antithrombin activity, when tested immediately or after incubation.⁹

Discussion. The results given in the paper show that after the administration of alloxan sufficient to cause an experimental condition of severe diabetes in rabbits, the coagulability of the blood was very slightly influenced. The clotting time of whole blood and recalcified plasma were slightly prolonged but the other tests that supply more specific information as to the manner in which the mechanism of the coagulation process is influenced, gave normal results. Normal activity of thromboplastin and prothrombin was demonstrated by the normal prothrombin consumption test and the normal prothrombin time. No antithrombin or any other anticoagulant could be demonstrated and the

activity of fibrinogen appeared to be normal.

From these results one must deduce that the influence of alloxan diabetes on coagulation is not significant and the prolongation of the coagulation time is probably a non-specific effect, the nature of which remains to be investigated.

Summary. Blood of rabbits injected with doses of alloxan monohydrate sufficient to determine an experimental condition of severe diabetes shows a slight delay of the clotting time of both whole blood and recalcified plasma but not accompanied by any appreciable modification of other tests giving more analytical information of the clotting process. The slight hypocoagulability, which is probably non-specific, is not due to the hyperglycemia as it is not corrected by an amount of insulin sufficient to normalize transiently the blood sugar level.

TABLE I.

Relationship Between Blood Glucose Level, Clotting Time of Whole Blood, Prothrombin Consumption, Prothrombin Time, Clotting Time of Recalcified Plasma and Relative Concentration of the "Labile Factor" of Prothrombin, 7 Days After Intravenous Injection of Alloxan Monohydrate (200 mg per kilo body weight).

Rabbit No.	Blood glucose level, mg per 100 cc blood	Prothrombin time, sec.	Clotting time,* min.	Clotting time of recalcified plasma, sec.	Relative conc. of "labile factor," %	Prothrombin consumption	
						1 hr	2 hr
						sec.	
Control	117.4	6	12½	95	100	16	21
"	125.6	6½	11½	120	85	23	28
"	106.0	6	13	95	100	11	15½
"	112.8	7	11	115	95	10½	17
1	382.1	6½	15	120	100	13	21
2	122.4	6	12	135	90	12	18
3	402.7	7	17½	160	100	22	30
4	257.4	7	18	150	100	20	24
5	442.8	6	16½	150	80	10	16½
6	239.7	6½	20	165	100	14	19
7	108.3	6½	9½	95	100	11½	14
8	193.7	6	17½	120	95	17½	19
9	320.4	6	14	150	100	10	18
10	379.5	6	16	150	85	14	18½

* Arterial blood.

relative concentration of the "labile factor" and the antithrombin activity were determined according to technics recently described.^{8,9}

Results. Two rabbits out of the 10 investigated failed to exhibit any modification of their blood sugar level. This is not surprising as occasionally animals have been found by other investigators¹⁰ which resist the action of alloxan. The other 8 gave a typical response and the modification of their blood sugar level is presented in Table I. Two hours after injection the rabbits presented a transitory hyperglycemia followed, about 4 hours later, by a hypoglycemic phase which persisted until the eighth hour. On the second day the rabbits showed a sharp elevation of the blood sugar level, which reached its maximum level on the fourth day and then remained stationary. Correspondingly a marked glycosuria was noted.

Data showing the effect of alloxan on various constants of the clotting process are shown in Fig. 1 and Table I. The clotting time of

whole blood and that of recalcified plasma appeared to be slightly delayed without any appreciable difference between the fourth and the fourteenth day of experimental hyperglycemia. The difference between these animals and normal controls is small but consistent. As observed by Hecht in rats, transitory reduction of the hyperglycemia to normal with injection of 4 units of insulin per kilo weight had no effect on the behavior of the clotting time. The prothrombin time was normal in all treated and untreated animals. The prothrombin consumption, taken one and 2 hours after the completion of clotting, showed wide variations both in normal and treated animals, without any direct relationship to the blood sugar level. Determination of the relative concentration of the "labile factor" showed no difference between treated and untreated animals.

The possible presence of an anticoagulant was ruled out. The addition of half volume of whole blood and plasma of normal and alloxan treated animals gave a clotting time close to the arithmetical average of the two. The possibility of the presence of an antithromboplastin was excluded as both normal and treated rabbit plasmas gave similar curves when their prothrombin times were determined with thromboplastin of varying

⁸ Stefanini M., and Quick, A. J., *Fed. Proc.*, 1948, 7, 191.

⁹ Stefanini, M., *Proc. Soc. Exp. Biol. and Med.*, 1948, 67, 22.

¹⁰ Walpole, A. L., and Innes, J. R. M., *Brit. J. Pharm. and Chemiother.*, 1946, 1, 174.

TABLE I.
Sterilizing Dosage of Penicillin in Mice Infected
Intracerebrally with Streptococci.

Group	Strain	Daily dosage in units Mean of 5 passages		
		1-5	6-10	11-15
A	S ₂	360	540	780
A	S ₂	480	660	630
B	T ₃	>1860	>21,000	>30,000
B	T ₃	>1860	>21,000	>30,000
C	U ₁	>1860	>21,000	>30,000
C	U ₂	>1860	>21,000	>30,000

vals, usually the fifth, tenth, and fifteenth passages, for *in vitro* penicillin sensitivity by the whole plate method.

Fertile hens' eggs, after incubation at 37.5°C for 10-13 days, were infected in the chorio-allantoic space with 0.1 ml of an 18-hour broth culture of streptococcus. This was followed immediately by penicillin G through the same burr hole and at approximately the same depth. Initially, the doses arbitrarily assigned were 0.1, 0.2, and 0.3 units of penicillin per egg in 0.1 ml amounts. Usually 2 eggs were used for each dosage, and 2 as untreated controls. After twenty-four hours incubation all eggs were harvested through the air sac and chorio-allantoic fluid streaked onto blood agar plates. Growth from the egg that had received the largest amount of penicillin was inoculated into broth for the next serial passage. The penicillin dosage was adjusted for each passage, so that 2 eggs received the same amount, 2 approximately 25% more, and 2 approximately 25% less than that of the egg from which the culture had been obtained. *In vitro* sensitivity tests were performed at the fifth, tenth, fifteenth, twentieth, and twenty-fifth passage by the whole plate technique.

Results. The results in mice are given in Table I. It may be seen that for the 2 group A organisms no significant increase in the penicillin requirement for bacterial sterilization was found between the first and fifteenth passage in mice.

With group B and group C streptococci, a positive culture was obtained in every instance from the brain of animals receiving the

maximum dose of penicillin. A dosage of 30,000 units of penicillin was reached by the ninth passage. It was maintained at this level for the next 7 passages, as this was close to the toxic dose for mice. Positive cultures were also obtained from mice that had been infected with the parent strains and treated with 30,000 units of penicillin. For this reason, the means of the first to fifth and sixth to tenth passages in Table I should not be interpreted as demonstrating an increase in resistance to penicillin. While this may be true, our data do not prove it.

When the group A, B, and C organisms were tested by *in vitro* technics, there was no difference in sensitivity between the parent strains and those obtained after 15 passages in treated mice.

The mouse virulence of all 6 parent strains, the same strains after 15 passages in normal mice, and after 15 passages in penicillin treated mice, were essentially alike.

The results in eggs are given in Table II. For the group A organisms there was no appreciable difference in the amount of penicillin necessary to sterilize the chorio-allantoic fluid in the first and the twenty-fifth passage in both strains studied. In contrast, both group B and C organisms showed a progressively increased resistance to penicillin on serial passages in treated eggs. Approximately 30 to 40 times as much penicillin was required to sterilize the chorio-allantoic fluid for the group B strains on the twenty-fifth passage as was needed for the first. For the group C organisms this difference was 12 and 3 fold respectively. This apparent change in penicillin resistance was not reflected in the *in vitro* sensitivities as all 6 strains maintained their initial sensitivities throughout the 25 serial passages.

Conclusion. Two strains of group A streptococci failed to acquire resistance to penicillin after repeated exposures to sublethal amounts either in embryonated hens' eggs or in mice. With 2 strains each of group B and C organisms, however, in eggs there was a definite increase in penicillin resistance. In mice this same increase may have occurred,

Antibiotic Studies on Beta Hemolytic Streptococci. IV. Penicillin Resistance Induced in Mice and Embryonated Eggs.

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Induced *in vivo* penicillin resistance has been demonstrated in only a few species of bacteria. Miller and Bohnhoff¹ were able to induce penicillin resistance in a strain of meningococcus by serial passage in mice when the animals received sub-protective doses of penicillin. After 12 passages, the PD₅₀ was increased 100 fold, and the *in vitro* resistance was increased 50 fold. After 60 passages, 170 times as much penicillin was needed to protect 50% of the mice as was required for the first passage. Schmidt and Sesler² using two strains of pneumococcus demonstrated a 4 fold rise in penicillin resistance after 7 passages in mice that had been treated with penicillin. In previous reports from this laboratory,³⁻⁵ the development of *in vitro* resistance to penicillin was demonstrated for 28 strains of groups A, B, and C beta hemolytic streptococci. The group A organisms developed resistance very slowly, the group B relatively quickly, and the group C immediately. The maximum change induced in the group A organisms after 60 transfers was 17 fold, in group B, 190 fold, and in group C, 16 fold. Milzer⁶ and his coworkers have recently shown that patients who had received oral penicillin had resistant strains of beta hemolytic streptococci present in their nasopharynx. Typing and grouping were not made, nor were sensitivities determined on

the strains before therapy was started. It would be impossible, therefore to say that the organisms that developed resistance were the strains present at the outset.

Technic. A total of 6 strains of beta hemolytic streptococci, 2 each of groups A, B, and C were used for the present study. The same strains, designated S₂, S₃, T₃, T₅, U₁, and U₂, were used in our previous reports.³⁻⁵ All were obtained from patients who had received no penicillin therapy.

For mouse passage, 0.03 ml of an 18-hour culture of the organism in veal infusion broth containing 5% defibrinated sheep's blood was injected intracerebrally into each of 6 mice. The animals, in pairs, received 3 injections of penicillin G at 2½-hour intervals. Initially, the total doses arbitrarily assigned were 150, 300, and 450 units given subcutaneously in 0.2 ml amounts. Twenty-four hours after infection, a surviving animal for each of the penicillin dosages was sacrificed, and broth cultures made of the brain and cerebrospinal fluid. The positive culture from the animal receiving the highest quantity of penicillin was used for the next serial passage. The dosage schedule was adjusted for each transfer, so that two animals received the same amount, two a larger amount, and two a smaller amount than that of the mouse from which the culture had been obtained. These changes were approximately 25% for the group A, and up to 100% for the group B and C organisms.

Virulence studies were made on the parent strain, the parent strain after 15 passages in normal mice, and the same strain after 15 passages in animals that had received sub-protective doses of penicillin. The technic used for this was the same as that described in a previous communication.³ The parent and resistant organisms were tested at inter-

¹ Miller, C. P., and Bohnhoff, M., *J. Inf. Dis.*, in press.

² Schmidt, L. H., and Sesler, C. L., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 353.

³ Gezon, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 208.

⁴ Gezon, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 212.

⁵ Gezon, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 215.

⁶ Milzer, A., Kohn, K. H., and MacLeon, H., *J. Am. Med. Assn.*, 1948, **130**, 536.

hydrate) of the "resistant" strains isolated after treatment determined.

The present report is concerned with a study of the penicillin sensitivity of strains of group A hemolytic streptococci isolated from scarlet fever patients before, during, and following therapy in order to determine whether or not resistance to the antibacterial agent developed during short periods of administration of the drug by either the oral or parenteral route.

Materials and methods. The patients from whom the strains hemolytic streptococci described in this paper were obtained had clinical evidence of scarlet fever and were started on penicillin therapy† on admission to the hospital. The total dose of the drug ranged from 1,200,000 to 3,000,000 units intramuscularly or 3,000,000 to 15,000,000 units orally given over a period of 10 days, the interval between each dose being either 3 or 8 hours depending on the schedule employed. Blood levels of penicillin were determined on most patients twice (2nd + 9th days) during treatment and were therapeutically adequate in every instance.

Cultures of the throat and nasopharynx of all patients were made at the time of admission, every day during the 10 days of treatment, and every other day after therapy was discontinued (usually 11 days). If beta-hemolytic streptococci were found in the nasopharynx after the penicillin was stopped, daily culturing was resumed. Characteristic colonies of beta-hemolytic streptococci were isolated from blood-heart infusion-yeast-agar, reinoculated on the same medium and stored at 4°C. All of the strains were grouped by the precipitin technic,⁸ and their penicillin sensitivity determined by the method of Ram-

melkamp⁹ using a known penicillin sensitive strain of *strep. pyogene* (R-98) as control.

Results. 195 strains of group A beta-hemolytic streptococci isolated from patients before they were treated with penicillin were tested for sensitivity to the drug. Two of the strains were inhibited by 0.0313 unit of penicillin, 75 by 0.0156 unit, 115 by 0.0078 unit, and 3 by 0.0039 unit. This range of sensitivity is essentially in agreement with that previously reported for this organism.³

Although penicillin eradicated the beta-hemolytic streptococci from the nasopharynx of most patients with scarlet fever in 48 hours, positive cultures were obtained during treatment on 37 different occasions. The number of hemolytic streptococcal isolations, the day on which the organisms were recovered and their penicillin sensitivity are shown on Table I. These strains had all been in contact with penicillin at the time when their drug sensitivity was determined since they were isolated from cases in which the streptococci failed to disappear despite treatment or from individuals in whom they had disappeared and subsequently recurred probably as the result of exposure to other patients in the ward who still harbored the organisms in the nasopharynx. None of the strains showed a greater tolerance to penicillin than that of the cultures isolated at the time of admission to the hospital before any antibiotic therapy was administered.

Beta-hemolytic streptococci belonging to group A were isolated in 24 instances after penicillin was stopped, the organisms being recovered 1 to 34 days after therapy was halted. Six of these strains were inhibited by 0.0156 unit of penicillin, 16 by 0.0078 unit and 2 by 0.0039 unit. None were penicillin resistant and their presence in the nose or pharynx following a full course of antibiotic administration can probably be accounted for best on the basis of contamination resulting from intimate or remote contact with individuals who were in the early phase of treatment or who had just entered the hospital and had not yet received any penicillin.

Discussion. The development of resistant strains of bacteria during contact with various antibiotic agents in use at present is well-

† The penicillin used in this study was supplied by the Schenley Laboratories, Inc., New York City. The intramuscular penicillin used was the potassium salt of crystalline penicillin G. The oral tablets were crystalline penicillin G potassium salt buffered with calcium carbonate, 50,000 or 100,000 units each.

⁸ Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, 78, 127.

⁹ Rammelkamp, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1946, 62, 54.

TABLE II.
Sterilizing Dosage of Penicillin in Eggs Infected in the Chorio-Allantoic Space with Streptococci.

Group	Strain	Daily dosage in units Mean of 5 passages				
		1-5	6-10	11-15	16-20	21-25
A	S ₂	0.19	0.13	0.17	0.24	0.28
A	S ₃	0.34	0.32	0.32	0.34	0.30
B	T ₃	1.70	2.80	1.76	3.60	5.60
B	T ₅	0.42	0.66	1.52	3.80	6.60
C	U ₁	0.25	0.18	0.54	0.52	0.66
C	U ₂	0.30	0.22	0.36	0.60	2.18

but we failed to demonstrate it through limitations of the method. The increasing penicillin resistance *in vivo* of the group B and C

streptococci was not associated with increasing resistance *in vitro*.

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Drug Sensitivity of Hemolytic Streptococci Isolated from Cases of Scarlet Fever Treated with Penicillin.*

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The development of resistance to penicillin has been observed to occur *in vivo* in strains of *Streptococcus viridans* and *Staphylococcus aureus* during therapy with this agent.^{1,2}

Although penicillin has had very wide use in the treatment of group A hemolytic streptococcus infections there have been no reports of the development of strains with lack of sensitivity to this drug which constituted therapeutic problems.

Available information³⁻⁵ would seem to

* This study was aided by a grant from Schenley Laboratories, New York City.

¹ Clark, W. H., Bryner, S., and Rantz, L. A., *Am. J. Med.*, 1948, **4**, 671.

² Spink, W. W., and Ferris, V., *J. Clin. Invest.*, 1947, **26**, 379.

³ Watson, R. F., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 65.

⁴ Meads, M., Ory, E. M., Wilcox, C., and Finland, M., *J. Lab. and Clin. Med.*, 1945, **30**, 723.

⁵ Rantz, L. A., Randall, E., Spink, W. W., and Boisvert, P. J., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 54.

indicate that *Strep. pyogenes* (group A) as encountered in the general population is almost uniformly highly susceptible to penicillin. Hirsch *et al.*,⁶ however, have reported the isolation of a strain of this organism which was susceptible only to 0.625 units of penicillin from a patient ill with scarlet fever who had not received any antibiotic therapy. Recently, Milzer *et al.*,⁷ reported the occurrence of penicillin-resistant beta hemolytic streptococci in the throat cultures of children with rheumatic fever treated prophylactically with 100,000 units of penicillin orally each day for at least 4 months. Although these strains were resistant to 10 units of penicillin following the period of prophylaxis, none of the streptococci present prior to chemotherapy were available for comparison; nor was the serologic group (group specific carbo-

⁶ Hirsch, H. L., Rotman-Kavka, G., Dowling, H. F., and Sweet, L. K., *J. A.M.A.*, 1947, **133**, 657.

⁷ Milzer, A., Kohn, K. H., and Mac Sears, R., *J. A.M.A.*, 1948, **136**, 536.

some way to anoxia of the erythropoietic cells of the bone marrow. Because in contrast to secondary polycythemia the percentage oxygen saturation of the arterial blood is normal,¹ the application of this theory to polycythemia vera requires that anoxia be produced by circumstances local to the bone marrow. Reznikoff, Foot and Bethea² have described a possible cause of local bone marrow anoxia in certain cases of polycythemia vera in marked narrowing and fibrosis of the arteries and arterioles of that organ. Brilliant support for this concept derives from the recent experiments of Schafer³ with dogs rendered hypertensive and polycythemic by proprioceptor depressor neurotomy.

In other cases of polycythemia vera the white blood cell count in the peripheral blood may be elevated with evidence of myeloid immaturity. Likewise, in the bone marrow myeloid hyperactivity and immaturity may be found.⁴ Splenomegaly is prominent and the disease process may evolve sooner or later into anemia associated with the features of myelogenous leukemia, non-leukemic myelosis, or even of osteosclerotic anemia.^{5,6} Castle⁷ has suggested that in this splenomegalic type of polycythemia vera the hyperactivity of the myeloid cells of the bone marrow causes a reduction in the oxygen available to the erythropoietic cells, either because of competitive utilization of the available oxygen by the myeloid cells or because of their mechanical interference with the vascular supply of the erythropoietic cells.

This paper reports an attempt to confirm the validity of this theory by a direct measure of the oxygen saturation and tension

of samples of blood removed from the sternal marrow cavity.

Methods. Two sets of investigations were carried out. In the first study conducted in 1941 5 cc of blood were withdrawn by gentle suction from the sternal marrow cavity. Thereafter, using conventional methods for handling the sample under oil in an ice bath and employing Wintrobe's dry oxalate mixture as anticoagulant, the carbon dioxide and oxygen contents of the sample as well as its oxygen capacity were determined in duplicate by the Van Slyke manometric combined method⁸ using the 0.5 cc technic. In several instances the pH of the marrow blood was determined anaerobically by the glass electrode method using the Beckman instrument.

In the second study begun in 1947 blood samples were obtained by sternal puncture without suction. A sterile 16 gauge needle was washed with sterile heparin solution, the stylet was placed in position and sternal puncture was performed as usual. The patient was then turned on his side so that the needle sloped downward away from its point. The stylet was withdrawn and in many instances blood began slowly to fill the hub of the needle. Whenever no blood appeared, gentle probing with a long stylet usually started a flow. The tip of a 22 gauge needle attached to a tuberculin syringe was then introduced into the hub of the sternal puncture needle and the blood carefully sucked up as it welled into the hub of the needle. The dead space in the needle and the syringe was completely filled with heparin solution, 1 mg per cc.

The blood samples so obtained varied from 0.6 to 1.0 cc in volume. They were handled as described by Roughton and Scholander⁹ except that a metal bead replaced the drop of mercury for mixing the blood and heparin solution. Peripheral arterial and venous blood samples were taken immediately after the sternal puncture, using the technic of collection and storage employed with the

¹ Hitzengerger, K., *Z. f. klin. Med.*, 1934, **126**, 495.

² Reznikoff, P., Foot, N. C., and Bethea, J. M., *Am. J. Med. Sci.*, 1935, **180**, 753.

³ Schafer, P. W., *Ann. Surg.*, 1945, **122**, 1098.

⁴ Zadek, I., *Ergebn. d. ges. Med.*, 1927, **10**, 355.

⁵ Rosenthal, N., and Bassen, F. A., *Arch. Int. Med.*, 1938, **62**, 903.

⁶ Vaughan, J. M., and Harrison, C. V., *J. Path. and Bact.*, 1939, **48**, 339.

⁷ Castle, W. B., Chapter on Blood, in *Pathologic Physiology and Mechanisms of Disease*, W. A. Sodeman, editor, in press.

⁸ Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

⁹ Roughton, F. J. W., and Scholander, P. F., *J. Biol. Chem.*, 1943, **148**, 541.

TABLE I.
Penicillin Sensitivity of Group A Beta-hemolytic Streptococci Isolated During Penicillin Therapy.

Penicillin sensitivity*	Route of penicillin† administration	Day of treatment and No. of strains of strep.										Total isolations
		2	3	4	5	6	7	8	9	10		
.0156	IM	3	1	1	1	3	1	2	2	1		15
	PO	1	0	0	0	0	0	0	0	0		1
.0078	IM	3	1	2	0	0	0	1	1	2		10
	PO	2	1	2	0	1	2	1	1	0		10
.0039	IM	1	0	0	0	0	0	0	0	0		1
	PO	0	0	0	0	0	0	0	0	0		0
Total		10	3	5	1	4	3	4	4	3		37

* Quantity which inhibited growth.

† IM—intramuscular; P.O.—oral.

known. The circumstances of the appearance of sulfonamide resistant group A beta-hemolytic streptococci in the Armed Forces has been reviewed elsewhere.¹⁰ The possibility that a similar situation might arise as a result of the extensive use of penicillin has been anticipated but not observed clinically.

Despite the failure of group A beta-hemolytic streptococci to develop proved penicillin resistance *in vivo* during treatment, there is good evidence that this phenomenon can be produced with ease *in vitro*. Weinstein and Tsao¹¹ made a number of strains of this organism 4 to 32 times more resistant to penicillin by frequent subculture in media containing increasing amounts of the drug; this resistance was only temporary, however,

and was made to disappear by frequent subculture in antibiotic-free media. Gezon,¹² using a slightly different technic, reported similar results.

Summary. (1) Studies of the penicillin sensitivity of group A hemolytic streptococci isolated from scarlet fever patients before, during, and following penicillin therapy indicate that, with the usual "short" courses of treatment, drug-resistant strains of the organism do not appear.

(2) The likelihood of the development of penicillin resistance in cases of streptococcal infection treated with this agent appears to be very remote.

(3) No penicillin-resistant strains of *Strep. pyogenes* (group A) were recovered in any instance before antibiotic therapy was started.

¹⁰ Hartman, T. L., and Weinstein, L., *New Eng. J. Med.*, 1948, **238**, 560.

¹¹ Weinstein, L., and Tsao, C. C. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 598.

¹² Gezon, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 208.

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Oxygen Saturation of Sternal Marrow Blood with Special Reference to Pathogenesis of Polycythemia Vera.*

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There are two main theories of the patho-

genesis of polycythemia vera. The older theory considers that the disease is neoplastic in nature. The other theory supposes that polycythemia vera, like most forms of experimental and clinical polycythemia, is due in

* The expenses of this investigation were defrayed in part by the J. K. Lilly gift to the Harvard Medical School.

† Deceased.

moderately anemic, ranged from 79.0 to 99.2 with a mean of $88.6 \pm 5.86\%$. The oxygen capacity of the arterial blood of these subjects ranged from 15.0 to 21.6 with an average of 18.8 ± 1.99 volumes %.

Polycythemia Vera. In the first study, 17 determinations of the oxygen saturation of the sternal marrow blood of 13 patients with polycythemia vera gave readings between 70.1 and 83.4 with a mean of $76.8 \pm 3.29\%$. Of these, 7 determinations were made on 6 patients with normal white blood cell counts in the peripheral blood (5,000 to 12,900 per cu mm. The results showed values between 78.8 and 83.4 with a mean of $80.4 \pm 1.64\%$. Ten determinations made upon 7 patients with elevated white blood cell counts in the peripheral blood (17,300 to 40,000 per cu mm) showed values of 70.1 to 78.9 with a mean of $75.0 \pm 2.69\%$.

In the second study the values obtained for the oxygen saturation of the marrow blood of 10 patients with polycythemia vera ranged from 80.3 to 93.0 with a mean of $87.3 \pm 4.49\%$. In the 3 patients with normal white blood cell counts, the corresponding values were 91.7, 88.5 and 81.7% respectively. The percentage oxygen saturation of the arterial blood samples was within normal limits in all the patients included in this study as well as in 8 other patients with polycythemia vera that exhibited elevated arterial oxygen capacities ranging from 22.8 to 33.0 volumes %.

Secondary Polycythemia. Including the observations from both studies 7 determinations of the percentage oxygen saturation of the marrow blood were made in 6 patients with polycythemia secondary either to cardiac or pulmonary disease. In 5 of these patients the oxygen capacity of the arterial blood was elevated, ranging from 22.7 to 30.7 volumes %. The other patient showed a normal oxygen capacity of the arterial blood because of previous venesections. However, in all the arterial blood was definitely unsaturated and carried only from 66.3 to 87.8% of its oxygen capacity. The oxygen saturation of the sternal marrow blood in all instances likewise fell below normal limits with extremes of 61.8 and 74.8%.

Anemia. Including observations made in both studies the percentage oxygen saturation of the blood in the sternal marrow of 12 patients with chronic anemia due to various causes was determined. The oxygen capacity of the marrow blood of these patients ranged from 6.0 to 14.2 volumes %. Excluding one patient, with the extremely low value of 58.4%, the oxygen saturation of the marrow bloods ranged from 72.9 to 94.5 and averaged 81.3 ± 6.34 . In the 8 patients of the second study in which the determination was made the oxygen saturation of the arterial blood samples was likewise normal.

Leukemias and Myeloid Metaplasia. In the first study the oxygen saturation of the marrow blood of 7 cases of leukemia and of myeloid metaplasia was determined, with results ranging from 50.0 to 73.7%. The average value was $65.8 \pm 7.8\%$. However, in the second study the oxygen saturation of the marrow blood of 4 patients ranged between 78.5 and 91.1%, indicating no significant departure from normal values.

Discussion. The results obtained and plotted in Fig. 1 do not in general demonstrate a lowered percentage oxygen saturation of sternal marrow blood in polycythemia vera. Also in the few instances in which, in the first study, oxygen tensions were calculated from the oxygen saturation and the pH of the sternal marrow blood according to the methods of Bock and his associates¹¹ no significant differences between the controls and the patients with polycythemia vera were demonstrated. In the patients with polycythemia secondary to pulmonary or cardiac disease, the percentage oxygen saturation of the marrow blood was distinctly reduced below that of the normals and convalescent controls. However, this was presumably due to the demonstrated unsaturation of the arterial blood.

It is important to note that even in the cases of anemia, where it is generally acknowledged that an anoxic stimulus to blood forma-

¹¹ Bock, A. V., Dill, D. B., Hurxthal, L. M., Lawrence, J. S., Coolidge, T. C., Dailey, M. E., and Henderson, L. J., *J. Biol. Chem.*, 1927, 73, 749.

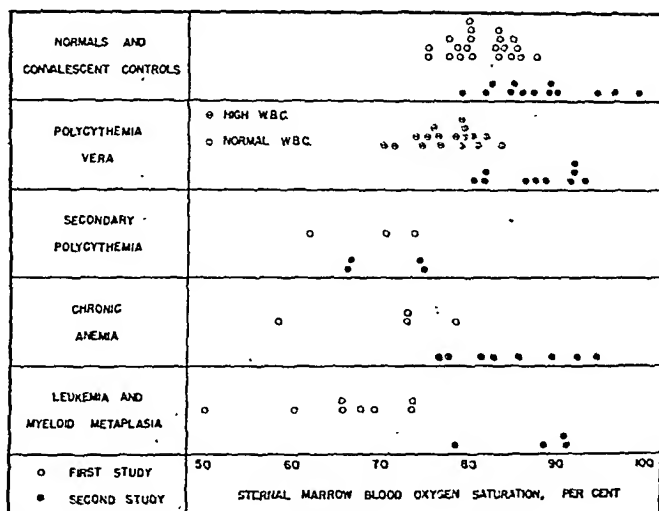


Fig. 1.

Percentage oxygen saturation of samples of blood removed by needle puncture from the sternal marrow cavity of normal subjects, convalescent control patients and patients with polycythemia vera, secondary polycythemia, chronic anemia, and leukemia or myeloid metaplasia. Each symbol represents the result of a single determination. The open dots indicate results from the first study; the solid dots those from the second. The results of the first study on patients with polycythemia vera are divided into two groups, according to whether the white blood cell count of the peripheral blood was normal, 5,000 to 12,000 per cu mm (open dots divided vertically) or high, 17,300 to 40,000 per cu mm (open dots divided horizontally).

marrow blood. Duplicate determinations were made by each of two operators of the oxygen content of the blood samples by the Roughton-Scholander micro method⁹ employing 0.04 cc of blood for each analysis. Appropriate allowance was made for the dilution produced by the known volume of heparin solution in the dead space in the needle and syringe. Oxygen capacity was determined by measuring the hemoglobin content of a 0.1 cc sample of blood in an Evelyn photoelectric colorimeter using a factor of 2.58.¹⁰ Control observations were made on the same sample of blood by the Roughton-Scholander, the Van Slyke and photoelectric methods respectively. The results in three such experiments on blood samples with oxygen capacities of 17.3, 17.8 and 18.9 displayed close agreement, the greatest differences being 0.2, 0.3 and 0.4 volumes % respectively. Other control observations on a dummy set-up using partially reduced blood showed no significant

exposure of the blood in the hub of the sternal puncture needle to the air before it was sucked into the collecting tuberculin syringe. The accuracy of the Roughton-Scholander technic in our hands when tested on atmospheric air was only slightly beyond the limits claimed for the method by its authors.

Results. The results of the observations are plotted in Fig. 1.

Controls. In the first studies the oxygen saturation of 21 marrow bloods derived either from normal subjects or from convalescent hospital patients ranged from 75.0 to 87.4 with a mean of $81.1 \pm 3.49\%$. The oxygen capacity of the sternal blood samples from these subjects ranged from 17.3 to 23.6 with an average of 19.8 ± 1.75 volumes %. The pH of the marrow blood in the 8 samples studied ranged from 7.45 to 7.54.

In the second study the oxygen saturation of 13 marrow bloods derived from convalescent hospital patients who were in 2 instances

Incidence of Mammary Tumors in Castrate and Non-Castrate Male Mice Bearing Ovarian Grafts.*

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Although extensive investigations have been carried out determining the state of function of ovaries grafted to castrate, non-castrate and cryptorchid male animals (for review articles see ^{1,2,3}), there appear to be only 2 reports of the successful production of mammary carcinoma in male mice by this surgical procedure. Murray⁴ observed mammary tumors in 7.1% of 210 dba castrate male mice bearing intraperitoneal ovarian grafts while de Jongh and Korteweg⁵ report the development of mammary carcinoma in 9 of 16 male mice similarly treated. Since it has been subsequently established that the rodent liver very effectively inactivates estrogenic substances carried through the portal circulation^{6,7} and that after castration the adrenals of male mice of at least one strain may produce sufficient estrogen for the development of mammary cancer,⁸ it was felt worth while to reinvestigate this problem. The data presented here represent a comple-

tion and extension of those previously reported in preliminary form.⁹

Materials and Methods. All mice used in these experiments were F₁ hybrids between the A and Z (C₃H) stocks maintained in this laboratory. As virgin female mice of the reciprocal A × Z cross exhibit a different incidence of mammary carcinoma as well as a different average age of tumor development,¹⁰ approximately equal numbers of animals of the two crosses were employed in each of the groups in these experiments. All surgical procedures were carried out soon after weaning, i.e., when the mice were 4 to 6 weeks of age, after which the animals were housed in wooden boxes, fed Purina Fox Chow *ad libitum*, and inspected once a week for the appearance of subcutaneous tumors. All such tumor masses were studied microscopically.

The method of ovarian transplantation was the same as that previously employed in female mice,¹¹ consisting of the removal of the ovaries from their encompassing ovarian sac and inserting them deep into the tissue of the axilla through a small skin incision. In our hands this method has proven superior to intraperitoneal implantation in mice since aseptic technics need not be employed as is necessary for intraperitoneal implantation, and the danger of portal vascularization is obviated. From the data obtained with ovarian transplantation into ovariectomized female mice, it would appear that ovaries grafted in this manner function very similar to those remaining in place within the abdomen. Castration was carried out via a transcrotal approach and was done at the

* Assisted by grants from the Minnesota Division of the American Cancer Society, the Citizens Aid Society of Minneapolis, The Graduate School Cancer Research Fund of the University of Minnesota, and the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

¹ Moore, C. R., and Price, D., *Am. J. Anat.*, 1932, **50**, 13.

² Smelser, G. K., *Physiol. Zool.*, 1933, **6**, 396.

³ Gardner, W. U., *Endo.*, 1935, **19**, 656.

⁴ Murray, W. S., *J. Cancer Res.*, 1928, **12**, 18.

⁵ de Jongh, S. E., and Korteweg, R., *Acta brev. Neerland.*, 1935, **5**, 126.

⁶ Zondek, B., *Skandinav. Arch. F. Physiol.*, 1934, **70**, 133.

⁷ Golden, J. B., and Severinghaus, E. L., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 361.

⁸ Wooley, G., Fekete, E., and Little, C. C., *Endo.*, 1941, **28**, 341.

⁹ Huseby, R. A., Smith, F. W., and Bittner,

J. J., *Cancer Research*, 1946, **6**, 494.

¹⁰ Bittner, J. J., and Huseby, R. A., *Cancer Research*, 1946, **6**, 235.

¹¹ Huseby, R. A., and Bittner, J. J., *Proc. Fourth Inter. Cancer Res. Congress*, 1947, in press.

tion exists, lowered percentage oxygen saturation of the marrow blood was not demonstrable in the present investigation. This result suggests that the failure to find decreased oxygen saturation in the marrow blood of the patients with anemia, and conceivably of those with polycythemia vera, may have been due to failure to obtain samples of blood that truly reflect the environmental conditions of the erythropoietic cells of the bone marrow. Penetration by the needle of the rigid bony cortex surrounding the marrow quite possibly creates a fundamental disturbance of the local pressure relations which, together with the inevitable injury to marrow vessels, may cause a totally abnormal situation with respect to local blood flow.

The values for the percentage oxygen saturation of the marrow blood in all patients tested, with a single exception, lay between those of the peripheral arterial and venous blood. Therefore, in the observations reported here as in those of Grant and Root¹² using a somewhat similar technic in dogs, it would appear that samples containing variable proportions of arterial and venous blood were obtained upon marrow puncture. The fact that, in general, in the first study the oxygen saturations of the marrow bloods were lower than in the second suggests that the suction employed in the first study caused a greater proportional backflow of blood from the venous side of the marrow circulation than occurred in the second study in which suction was not used. Because the venous blood leaving the marrow presumably reflects its total metabolic activity, the samples obtained by suction may have more nearly represented its rate of oxygen consumption than did those obtained without suction in the second study.

In the light of this possibility it is interesting to note that there is a suggestion in the first study that the patients with high peripheral white blood cell counts, that is, the cases with myeloid overactivity, tended to show lower percentage oxygen saturations of the marrow blood than did patients with

normal or low white blood cell counts. Another feature of interest in the first study is the consistently low values for percentage oxygen saturation obtained in the cases of leukemia and of myeloid metaplasia. Because increased oxygen consumption by the leukocytes of the blood after withdrawal of the sample from the bone marrow could not be demonstrated, these findings suggest that increased oxygen utilization relative to the blood supply was actually occurring in the bone marrow in these patients.

Summary. An attempt was made to measure the stimulus to erythropoiesis in the human bone marrow by estimating the percentage oxygen saturation of blood removed from the sternal marrow cavity through a needle inserted through the cortex as in the performance of a sternal needle biopsy. By this means, blood samples were collected without effective contact with air and their oxygen content, capacity and in some instances pH were determined. In general no significant differences were demonstrated between normals, convalescent controls, anemic patients, and patients with polycythemia vera. In patients with secondary polycythemia, the percentage oxygen saturation of the blood removed from the bone marrow was relatively reduced, probably entirely as a result of the manifest unsaturation of the arterial blood. In some patients with leukemia and with myeloid metaplasia and in some patients with polycythemia vera with evidence of excessive myeloid activity, the data suggest an increased local oxygen utilization relative to the blood flow in the bone marrow. However, it was concluded that the techniques used were not adequate to demonstrate an anoxic stimulus to increased erythropoiesis even in the marrow of patients with chronic anemia, possibly because of the difficulty of obtaining blood samples satisfactorily representative of the undisturbed environment of the erythropoietic cells.

We desire to express our appreciation to Dr. Benjamin Alexander for permission to carry out the determinations on several of the patients in the second study.

¹² Grant, W. G., and Root, W. S., *Am. J. Physiol.*, 1937, 150, 618.

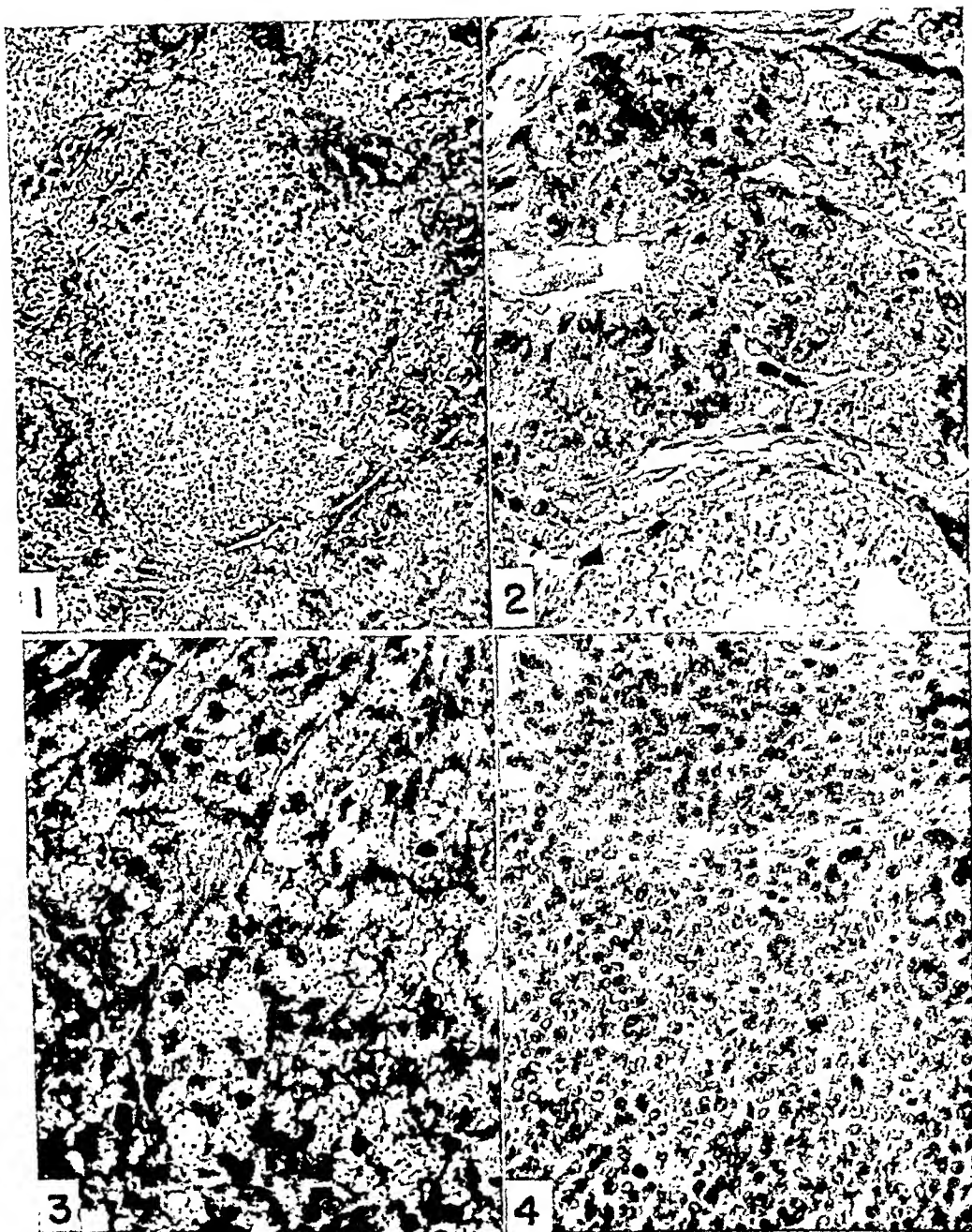


FIG. 1. An area of an ovarian graft carried in a castrate male mouse showing a small corpus luteum. Bouin's fixation and hematoxylin eosin stain. Mag. $\times 140$.

FIG. 2. A group of interstitial cells in an ovarian graft from an ovariectomized female. Note the numerous granules in the cytoplasm and the absence of vacuolization. Resorcinol-formol fixation and Altmann-Bensley stain. Mag. $\times 800$.

FIG. 3. An area of interstitial cells in an ovarian graft carried in a castrate male. Note the extensive vacuolization of the cytoplasm and the paucity of granules. Fixation and staining as in Fig. 2. Mag. $\times 800$.

FIG. 4. An area from a granulosa cell tumor developing in an ovarian graft carried in a non-castrate male. Fixation and staining as in Fig. 1. Mag. $\times 150$.

TABLE I.

Summary of Tumor Data from Castrate and Non-castrate Hybrid Males Transplanted with Ovaries. For comparison similar data is given for intact female and ovariectomized ovarian transplanted females of the same hybrid cross.

Group	No. animals/ No. cancerous	% cancerous	Avg age	
			Of cancer development, mo.	Non-cancerous deaths, mo.
Mammary tumors in F ₁ hybrid males bearing transplanted F ₁ hybrid ovaries.				
Castrate	31/27	87.1	11.7	16.9
Non-castrate	39/1	2.5	19.4	27.0
Mammary tumors in normal female mice of the A × Z reciprocal crosses. ¹⁰				
Virgin				
Breeding				
Stock	% cancerous	Avg age of cancer, mo.	% cancerous	Avg age of cancer, mo.
AZF ₁	92.5	15.2	98.0	9.7
ZAF ₁	73.2	19.1	97.6	10.1
Average	82.9	16.9	97.8	9.9
Mammary tumors in ovariectomized female mice of the A × Z crosses bearing transplanted hybrid ovaries. ¹¹				
Stock	% cancerous	Avg age of cancer, mo		
AZF ₁	92.3	15.9		
ZAF ₁	48.1	21.1		

same time as the ovarian transplantation.

Results. Data dealing with mammary tumor development are summarized in Table I. From these it is evident that the presence of the normal testes profoundly affects the development of mammary cancer in male mice bearing ovarian grafts. It is evident, further, that castrate male mice with ovaries develop carcinomas of the mammary gland at a significantly earlier age than do normal virgin females or ovariectomized females bearing ovarian grafts. In fact, in this respect, these castrate male mice bearing ovaries more closely resemble breeding females of this hybrid cross than they do virgin females. This was further emphasized by the fact that there was no difference in the male mice of the two reciprocal crosses as far as either the incidence of tumors or the average age at which these tumors developed.

The ovaries of all the castrate animals that developed mammary tumors and of 12 non-castrate males sacrificed between 362 and 501 days of age were studied employing routine histological procedures. To demonstrate finer cytological details, 2-month-old ovarian grafts carried in castrate and non-

castrate males and in ovariectomized female mice were fixed in resorcinol-formol,¹² sectioned at 2 micra, and stained according to the Altmann-Bensley method.

The appearance of the grafts in ovariectomized females was essentially that of normal ovaries. Those recovered from non-castrate males differed mainly in that corpora lutea were absent, and thus the size of these transplants was smaller than that of the transplants carried in ovariectomized females. In the grafts of all mice of this group that were studied, however, numerous normal appearing follicles were present. These ranged in size from primary follicles to ones of moderate size possessing well formed antra. The interstitial cells of these ovaries were somewhat atypical in that with a routine hematoxylin-eosin stain their cytoplasm appeared moderately hypertrophied and more vacuolated than is normal for this strain of mouse. However, the over-all size of the "interstitial organ" was not particularly excessive. In all 20 non-castrate mice sacrificed for histological study, well maintained ovarian

¹² Huseby, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1946, 61, 122.

TABLE II.

Development of Uterine and Vaginal Grafts and Mammary Glands in Mice 2 Months After Ovarian Transplantation.

Ovaries transplanted to	Uterine grafts	Vaginal grafts	Mammary glands
Ovariectomized females	Developed. Avg 6.0×4.3 mm	Cyclic	Developing
Non-castrate males	Developed. Avg 5.3×3.9 mm	No evidence of estrogenic stimulation*	Little or no development
Castrate males	Developed. Avg 8.0×7.0 mm	Non-cyclic cornified	Developing

* There may be a beginning mucinification of the epithelium suggesting estrogen-androgen cooperation.

castrate males by grafting bits of uteri and vaginae into the subcutaneous tissue of such mice. As a preliminary experiment, the uteri and vaginae of young F_1 hybrid females were removed, each uterine horn was split longitudinally, and each vagina was cut transversely into two narrow segments. Single uterine and vaginal fragments were then transplanted into the subcutaneous tissue (in either the axilla or groin) of several intact male and female F_1 hybrid mice. Two months later the recipient animals were sacrificed, and the grafts studied histologically.

The uterine grafts carried in female mice formed large thin-walled cysts lined with typical uterine epithelium. Because of the pressure exerted on the cyst wall by the accumulating fluid, the epithelial cells were generally rather low columnar or cuboidal in form. Only in areas of papillary infoldings were the characteristic tall columnar cells with significant amounts of underlying lamina propria found. In the intact male animals the uterine grafts had been reconstituted so as to form small cylindrical bodies very similar to the intact donor uteri before being split longitudinally. There was no tendency for fluid accumulation so that the normal uterine relationships were disturbed very little. Morphologically these grafts closely resembled uteri of castrate female mice except for a slightly "looser" appearance of the lamina propria.

Vaginal grafts were retained as cysts also. The epithelial lining seen in the grafts in the female animals faithfully reproduced that of the *in situ* vaginal epithelium, and the cyst lumen was filled with alternate layers of

leukocytes and desquamated cornified epithelial cells. On the other hand, the epithelium of the vaginal grafts maintained in intact male mice consisted entirely of two layers of epithelium similar to that found in castrate female mice and the luminal contents were devoid of cellular components. This method of uterine and vaginal transplantation showed promise then of shedding light on the state of function of ovarian grafts carried in male animals.

Groups of 8 weanling hybrid mice were set up as follows: non-castrate males, castrate males, and ovariectomized females. Into each animal was transplanted 2 ovaries, a vaginal, and a uterine fragment. Two months later the animals were sacrificed, the uterine and vaginal grafts measured (2 diameters at right angles to one another), and histological preparations of all grafts studied. A summary of these findings is given in Table II. It is evident from the size and histology of the uterine grafts in the non-castrate male group that the ovaries of these animals were producing estrogenic substances in appreciable amounts, although in all probability, in lesser quantities than produced by the ovaries of the castrate group. In view of this, the morphology of the vaginal grafts is of considerable interest. The epithelium of the grafts in the non-castrate animals was formed throughout by only 2 layers of cells much the same as in the vaginal grafts of intact male mice without ovarian transplants. The only indication of development was a tendency for the luminal layer of cells to be low columnar in form and to possess an increased amount of clear cytoplasm. This appearance

grafts were found.

The ovarian grafts recovered from castrate male mice differed from those of non-castrate males and of normal females. Although most reports of histological examination of ovarian grafts carried in castrate male animals, mainly in guinea pigs, record a complete absence of corpora lutea, not infrequently in the ovaries of these castrate male mice typical small corpora lutea were encountered (Fig. 1). The ovaries of F₁ hybrid females of the A × Z cross more closely resemble those of the Z parent in that corpora lutea are retained for a considerable time and thus normally this element makes up a large portion of the ovarian mass during the reproductive period in the animal's life.¹¹ It is evident, therefore, that although corpora lutea appeared not infrequently in the ovarian grafts of these castrate males, they were by no means as prevalent as in ovaries maintained in female animals. Conversely, however, the interstitial tissue of these transplanted ovaries was greatly hypertrophied and the cytoplasm of the individual interstitial cell possessed many large vacuoles. Follicles were numerous, appeared cytologically normal, but in general more large follicles with medium to large sized antra were evident than is the case in normal ovaries in female mice. In size these ovarian grafts considerably exceeded those of the non-castrate males and equaled or slightly exceeded those in ovariectomized females. Weights were not taken because of the difficulty of dissecting the ovaries completely free of surrounding connective tissue.

The detailed cytological study of the ovarian grafts revealed the follicular elements to be normal in all instances as were the cells of the large corpora lutea in the female grafts and of the small corpora lutea in the grafts of the castrate males. The interstitial cells of the 3 groups of ovaries differed greatly, however. In the female grafts studied, as well as in normal females of this cross, the cytoplasm of the interstitial cells was generally crowded with rather large, brilliantly fucsinophilic granules (Fig. 2). In the non-castrate males some cells possessed a large number of these granules while the cytoplasm of others was rather extensively vacuolated

with a loss of granulation. The cytoplasm of the interstitial cells of the ovaries in the castrate males was uniformly hypertrophied and extensively vacuolated with but few fucsinophilic granules remaining (Fig. 3).

These histological studies would suggest that the ovaries in the castrate males were subjected to a greater pituitary stimulation than were those in the non-castrate group.¹² In the majority of cases the adrenals of castrate tumor bearing animals were studied also, and no example of adrenal hyperplasia was noted.

In view of the great difference in the development of mammary carcinoma in the two groups of animals of this experiment, a histological study of their mammary glands was made employing the whole mount technic. The glands of the castrate males were found to be extensively developed with large ducts extending throughout the major portion of the various fat pads. In addition to ductular growth there were many areas of alveolar development, some of which formed typical pre-cancerous lesions while others appeared as more normal lobules in the early stages of secretion, *i.e.*, similar to those seen in normal female glands about the time of parturition. In some animals the mammary ducts were filled with a white, milk-like secretion. As has been observed by others, all 10 glands rarely develop in male mice receiving estrogenic stimulation. As contrasted to this picture, no definite example of mammary gland development was found in the non-castrate males. In some a few fine thread-like ducts could be seen extending a considerable distance into a fat pad, particularly in the number two position, but such development is not infrequent in normal male mice of this hybrid cross. Neither lateral buds nor alveoli were seen in these glands. Examination of the glands of the one non-castrate male that developed a mammary adenocarcinoma showed no more mammary growth than was seen in the other animals of the group.

It seemed possible to test the function of the well preserved ovarian grafts of non-

¹² Pfeiffer, C. A., and Hooker, C. W., *Anat. Rec.*, 1942, **83**, 543.

grafts of the animals receiving 0.15 γ of estrone generally exceeded those of the other 2 groups.

The study of the vaginal grafts and mammary glands suggest two major points. First, there is a considerable difference in the sensitivity threshold of these 2 tissues to estrone. Second, there would appear to be some antagonism between testicular hormone and estrone. To establish this latter conclusion with certainty, however, differences in adrenal function must be completely ruled out, although under the conditions of this experiment significant differences in this respect seem unlikely. Although the state of function of the pituitary does not appear to alter the vaginal response to estrogen,¹⁵ it has been found that mammary gland growth is dependent upon a functional pituitary gland (for review see ¹⁶). In order to be certain that the amounts of estrone injected had not caused a sufficiently severe hypophyseal suppression to inhibit mammary growth in the non-castrate males, the testes, ventral prostate, and seminal vesicles of each animal were studied. In all instances these organs were grossly and microscopically indistinguishable from those of normal intact males. Since, in our experience, doses of estrogens considerably lower than that required to cause "stunting" of the mammary gland produce easily demonstrable reduction in the production of pituitary gonadotropin, it would appear that in these estrone injected non-castrate mice pituitary suppression was not the factor responsible for the reduced response of the mammary glands.

The non-castrate males bearing ovarian grafts lived significantly longer than do virgin female mice of this hybrid cross (those lacking the milk agent so that no mammary cancer developed.¹⁰) It is, therefore, of interest to record that in 4 of these animals typical granulosa cell tumors developed in the area of the transplanted ovary (Fig. 4). These tumors developed when the mice were very old: 24.9, 25.4, 30.2, and 32.2 months of age.

In none of these 4 animals was there evidence of high estrogen levels since the mammae were no more developed than in other animals of the group.

Since it has been suggested that an increased pituitary gonadotropin level is responsible for ovarian tumor formation in the Biskind-Biskind type of experiment,^{17,18} a group of 35 F₁ hybrid males lacking the milk agent were castrated and transplanted with ovaries in the axillary position. The average life of these males was 24.6 months, but only two granulosa cell tumors were observed: at 19.3 and 23.3 months. Although these tumors developed somewhat earlier in life than did those in the non-castrate males, it is evident that no increase in the frequency of this tumor was brought about by castration. One of these tumors was transplanted to the subcutaneous tissue of other F₁ hybrid mice and was observed to grow rapidly in intact as well as in castrate males and in intact females. No evidence of estrogen production by these transplants could be found.

As there are no data available on the spontaneous occurrence of granulosa cell tumors of the ovary in normal female mice of this hybrid cross to use for comparative purposes, the ovaries transplanted to oophorectomized females of this strain¹¹ were reviewed. For this comparison only mice living to be 700 days of age or older were included. There were 18 female animals bearing hybrid grafts and living to an average age of 26.0 months available for study. In these only one atypical ovary possessing an overgrowth of granulosa cells was encountered (age 28.7 months). This is to be compared with the non-castrate male group bearing ovarian grafts in which 21 lived beyond 700 days of age (average age of death 28.0 months) and in which 4 typical granulosa cell tumors developed. Because of the small number of tumors observed and the somewhat longer average life of the male group, no statement as to the relative frequency of this

¹⁷ Biskind, M. S., and Biskind, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, **53**, 176.

¹⁸ Li, M. H., and Gardner, W. U., *Science*, 1947, **105**, 13.

¹⁵ Smith, P. E., *Am. J. Anat.*, 1930, **45**, 205.

¹⁶ Hulseby, R. A., Ball, Z. B., and Visscher, M. B., *Cancer Research*, 1945, **5**, 40.

TABLE III.

Development of Vaginal Grafts and Mammary Glands in Castrate and Non-castrate Male Mice After Injection of Various Doses of Estrone for 3 Weeks.

Amt of estrone		Vaginal mucosa	Mammary gland
.08 γ in 2 days twice per week	Castrate	Partial cornification with considerable mucinification	No development
	Non-castrate	No evidence of estrogen activity	No development
.05 γ per day	Castrate	Cornification with some areas of mucinification	Only 1 of 4 animals showed mammary development
	Non-castrate	Epithelial development mainly mucinification	Little or no development
.15 γ per day	Castrate	Completely cornified	Good mammary development
	Non-castrate	Cornification with some areas of mucinification	Little or no development

is suggestive of beginning mucinification which Korenchevsky and Hall¹⁴ have shown to result from the cooperative action of estrogens and androgens or progesterone. The vaginal grafts in the castrate male animals are also worthy of some description, for although they appeared extensively stimulated, there was no indication of estrous cycling. The epithelial histology was the same in all 8 grafts; however, there was considerable variation from area to area within each graft. While most areas showed complete or almost complete cornification, others exhibited only partial cornification with leukocytes passing between the epithelial elements. The lumina were filled uniformly with desquamated cornified epithelium mixed with some leukocytes, but with no indication of stratification as was seen in the grafts carried in normal female mice. This "mixed" vaginal histology is similar to that seen in female mice subjected to a constant stimulation with estrogen.

Although the difference in uterine and vaginal-mammary development in the non-castrate male mice might reflect only a difference in tissue sensitivity to estrogen, it might also reflect an antagonism between the two classes of sex hormones with regard to the vaginal and mammary epithelium. This

latter possibility was investigated by transplanting a group of hybrid male mice with uterine and vaginal fragments and allowing 2 weeks to elapse for vascularization of the grafts. Half of the mice were then castrated, and groups of castrate and non-castrate animals were injected with graded doses of estrone with 4 castrate and 4 non-castrate animals receiving each dose level. Injections were begun the day following castration in order to minimize the possibility of an increased estrogen production by the adrenals of the castrate animals. The estrone was injected subcutaneously in aqueous solution, the daily dose being equally divided between a morning and an afternoon injection. Animals at the lowest dosage level received 0.4 γ a day on 2 successive days and for 4 days of each week. The other groups received 0.05 and 0.15 γ daily 7 days a week. Injections were continued for 3 weeks, and the animals were autopsied within 6 hours of their last injection. A summary of the results is given in Table III. The data on the uterine grafts are not included as measurements on such a small number in each group could not be considered conclusive. It can be said, however, that at all dosage levels the uterine grafts became cystic and that there was no consistent difference in size between the grafts of castrate and non-castrate animals receiving the same amount of estrone, although the

¹⁴ Korenchevsky, V., and Hall K., *J. Path. and Bact.*, 1937, 45, 681.

onate concurrently. The possibility that the antagonism in the mammae is due to pituitary suppression resulting in mammary gland "stunting" seems to be ruled out in our experiment in which relatively small doses of estrogen and the androgen from functioning testes were employed.

Conclusion. Castrate male mice of the F_1 hybrid generation between the A and Z stocks bearing subcutaneous ovarian grafts developed mammary cancer more readily than do virgin female mice of this cross. Only 1 of 39 non-castrate males bearing ovarian grafts de-

velop mammary carcinoma as little mammary development occurred. Additional experiments seemed to indicate that the ovaries in the non-castrate male mice were producing an appreciable amount of estrogen although measurably less than those carried in castrate males. There appeared, also, to be an antagonism between estrogens and androgens as far as stimulation of the vaginal and mammary epithelium is concerned. Granulosa cell tumors occasionally occurred in ovarian grafts of old male and female mice of this hybrid cross.

16708 P

The Effect of 11-Desoxycorticosterone Acetate Upon the Glycosuria of Partially Depancreatized Rats.

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This study shows that large doses of 11-desoxycorticosterone acetate cause an exacerbation of the diabetes of partially depancreatized, force-fed rats.

Methods. Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they reached a weight of 300 g. Ten rats were depancreatized by the method of Ingle and Griffith.¹ After diabetes was established all of the animals were placed in

metabolism cages and were fed a medium carbohydrate diet made according to Table I. All of the animals were force-fed by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P. M.). The techniques and diets were modifications of those described by Reinecke, Ball and Samuels.² During the period of adaptation to force feeding, the amount of diet was increased gradually to prevent "food shock." A full feeding of 26 cc of diet per rat per day was reached on the 5th day.

The animals were kept at a temperature of 74° to 78°F and a humidity of 30 to 35% of saturation. Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) and were preserved with thymol. Urine glucose was determined by the method of Benedict.³

The 11-desoxycorticosterone acetate (Cortate, Schering) was made up in sesame oil at 5 mg per cc. It was given by subcutaneous in-

TABLE I.
Medium Carbohydrate Diet.

Constituent	G
Cella flour (Chicago Dietetic Supply)	120
Osborne & Mendel salt mixture	40
Dried yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Vitamin K (2-methyl-1,4-naphthoquinone)	100 mg
Mazola oil	200
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
Water to make total of	2000 cc

¹ Ingle, D. J., and Griffith, J. Q., Chapter 16, *The Rat in Laboratory Investigation*, J. B. Lippincott Co., Philadelphia, 1942.

² Reinecke, R. M., Ball, H. A., and Samuels, L. T., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 44.

³ Benedict, S. R., *J. I. M. A.*, 1911, **57**, 1193.

type of tumor in the two groups seems justified.

It seems of value, also, to record the occurrence of ovarian tumors developing in the ovaries of the two parent strains when transplanted to ovariectomized F_1 hybrid females. There were 11 hybrid females bearing Z or C_3H ovaries living beyond 700 days of age, and in these one mixed ductular-granulosa cell tumor was found (age 24.2 months). In 13 hybrid females past 675 days of age and bearing strain A ovaries, one typical luteoma (23.5 months) and two granulosa cell tumors (22.5 and 25.4 months) were encountered.

Discussion. It has been previously recorded that castrate male guinea pigs bearing ovarian grafts exhibit a greater mammary gland development than do normal virgin females (for review see ²). The present experiments with mice show that castrate males with ovarian grafts developed mammary cancers earlier than do normal virgin females of this hybrid cross, in fact the average age of tumor development approached that seen in similar breeding females. Although Smelser² found some indication of cyclic pituitary function in castrate, ovarian grafted male guinea pigs, our experience with vaginal grafts in castrate, ovarian grafted male mice suggests that the gonadotropin stimulation from these male pituitaries is at all times such that sufficient ovarian estrogen is produced to extensively cornify the vaginal epithelium. Although the largest part of the epithelium of these grafts was completely cornified, areas were encountered in all in which leukocytes could be seen passing through an incompletely cornified epithelium. This "mixed" picture is consistent with that seen in vaginae subjected to long periods of continuous estrogen stimulation.

The failure of the mammal to develop in the non-castrate males bearing ovarian grafts is in agreement with the observations of Smelser in guinea pigs. It is, however, at variance with those of Gardner³ who employed intratesticular grafts in CBA mice. This may, of course, be a strain difference. However, Gardner's photomicrographs show consider-

able tubular damage in the testes due to the ovarian transplant. It seems possible that this might explain the difference since Smelser observed mammary development in cryptorchid guinea pigs bearing intranephric ovarian grafts in which the androgen production by the cryptorchid testes was adequate to maintain the secondary sex glands but in which tubular alterations were extensive because of the intra-abdominal position of the testes. Further experimentation is necessary to establish or reject such an explanation.

The results of the experiments reported here designed to test the function of the ovarian grafts in the non-castrate male animals would seem to indicate that significant amounts of estrogen were being produced, but that the quantities were smaller than those produced by the ovarian grafts carried in castrate males. Any estimation of relative amounts of estrogen production by the grafts in these two groups of animals seems unwarranted since the picture appears to be further complicated by antagonism between estrogen and androgen with regard to vaginal and mammary gland development in the non-castrate group.

An estrogen-androgen antagonism for both the vaginal and mammary epithelium seems fairly well established by the results of the last experiment reported here. At the lowest dose level of estrone, the vaginal grafts in the castrate males showed definite histological evidence of stimulation while in the non-castrate group no demonstrable epithelial development was seen. At the higher dose levels, the cooperative action of the two hormones as described by Korenchevsky and Hall¹⁴ is also clearly demonstrated. Robson¹⁹ has suggested such an antagonism, but since he used only smears to follow the vaginal response one does not know whether he observed true antagonism or the co-operative effects resulting in epithelial mucinification which would also produce a "negative" vaginal smear. Gardner²⁰ has also shown this antagonism for the mammary gland by injecting estradiol benzoate and testosterone propi-

¹⁹ Robson, J. M., *J. Physiol.*, 1937, 90, 15.

²⁰ Gardner, W. U., *Cancer Research*, 1946, 6, 493.

Inhibition of Brain Respiration by Ethyl Alcohol at Varied Temperature Levels.

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Jowett¹ has reported that given concentrations of ethyl alcohol produce less inhibition of rat brain respiration at 34°C than at 39°C. The experiments reported here were carried out in order to study the inhibitory effect of ethyl alcohol over a wider range of temperature and concentration than had hitherto been investigated.

Methods. The oxygen consumption of cerebral cortex slices from adult albino rats was measured *in vitro* by the direct method of Warburg at 37.7°C, 30°C and 20°C. The tissue slices were weighed on a micro torsion balance and transferred to respirometer vessels containing Ringer's solution² buffered to pH 7.25 with 0.01 M phosphate and containing 0.011 M glucose. The gas phase was oxygen. Ethyl alcohol in appropriate concentration, in one-tenth the total fluid volume, was added from the side arm of the vessels at the end of a 10 minute period of thermoequilibration. Rates of oxygen consumption are expressed as ml of oxygen consumed per mg initial dry weight (determined on aliquots) per hour (QO₂). Complete details of the experimental procedure have been given elsewhere.³

Results and discussion. The rates of oxygen consumption of rat brain slices in the presence of various concentrations of ethyl alcohol at 37.7°C, 30°C and 20°C are given in Table I. The values of QO₂ at 37.7°C are the means of 4 determinations while those at other temperatures are single determinations. Additional values of QO₂ (not given in the table) at 37.7°C for the period 120-180 minutes after addition of alcohol are 11.10

at 0.95% alcohol, 10.43 at 2.85%, 6.91 at 4.75% and 2.16 at 6.65%.

The inhibition of brain respiration produced by ethyl alcohol has been reported by Jowett¹ to become progressively greater with time. It may be seen from the table that the progressive inhibition is more marked at 37.7°C than at 30°C. At 20°C this decrease in rate of oxygen consumption with time does not occur within 3 hours. At all of these temperatures the QO₂ of control brain slices, without addition of alcohol, remains constant for at least 5 hours.

In order to minimize the effect of the progressive inhibition in the presence of alcohol, the QO₂ for the period 120-180 minutes after addition of the inhibitor has been selected for purposes of comparison. It is apparent that the minimum effective inhibitory concentration of alcohol increases with decrease in temperature. It is approximately 2.5% at 37.7°C, 6% at 30°C and 7% at 20°C. The percentage inhibition produced by a given concentration of alcohol is greater at 37.7°C than at the lower temperatures. With 9.5% alcohol, for example, the inhibition is 91% at 37.7°C., 64% at 30°C and 21% at 20°C. The absolute level of oxygen consumption in the presence of 9.5% alcohol is remarkably similar at the 3 different temperatures.

At 37.7°C the inhibitory effect of higher concentrations of alcohol was studied. With 23.73% alcohol the QO₂ was 0.93 and further decrease did not occur over a period of 3 hours. The oxygen consumption was completely abolished by 47.5% alcohol. There is thus a large range of alcohol concentration, from about 8% to well over 25%, within which the rate of oxygen consumption is approximately constant. This fraction of oxygen consumption of brain, amounting to about 10% of the control rate at 37.7°C, has been

¹ Jowett, M., *J. Physiol.*, 1938, **92**, 322.

² Dickens, F., and Greville, G. D., *Biochem. J.*, 1935, **29**, 1468.

³ Fuhrman, F. A., and Field, J., 2nd., *J. Pharm. Exp. Therap.*, 1943, **77**, 229.

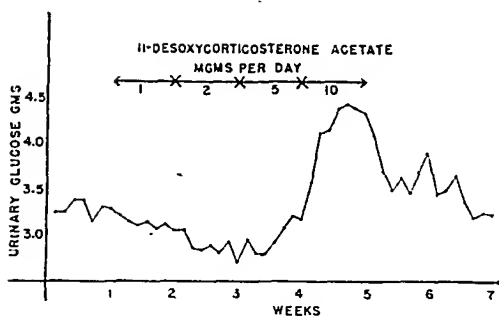


FIG. 1.

Effect of 11-desoxycorticosterone acetate upon the average level of urinary glucose for 10 partially depancreatized rats.

jection in divided doses each morning and late afternoon.

Experiments and results. Ten mildly diabetic rats were observed for periods of several weeks prior to the beginning of the experiment. All of the rats received doses of 1, 2, 5 and 10 mg of 11-desoxycorticosterone acetate for periods of 7 days per dose. At the completion of the injection periods the rats were observed during an additional control period of 14 days before the experiment was terminated. Several days elapsed between the beginning of the injection period for each rat and those tested subsequently so that possible unknown variables related to time were less likely to cause fortuitous shifts in the average level of glycosuria. This is an important control for on many occasions we have observed small but significant spontaneous shifts in the level of glycosuria in most or all of a series of diabetic animals under rigid experimental conditions and constant food intake. The results are shown in Fig. 1. During the admin-

istration of 1 and 2 mg of the steroid per day there was an average decrease in the level of urinary glucose representing a change in 7 of the 10 experimental animals. When 10 mg per day of 11-desoxycorticosterone acetate were given, there was an exacerbation of the glycosuria in each of the 10 rats which decreased to pre-injection levels following the withdrawal of the hormone.

Discussion. The decrease in glycosuria which occurred in most of the rats during the administration of 1 and 2 mg per day of the steroid may have been due to the compensatory suppression of the secretion of the 11-oxy steroids by the adrenal cortices according to the hypothesis of Selye and Dosne.⁴ However, the changes were not sufficiently striking to permit the definite conclusion that the decrease was caused by the administration of the steroid. The exacerbation of glycosuria during the injection of 10 mg per day of the steroid was clearly significant and supports the conclusion that 11-desoxycorticosterone does affect carbohydrate metabolism when given in large doses.

Summary. Ten mildly diabetic force-fed rats showed an average decrease in the level of urinary glucose during the administration of 1 and 2 mg of 11-desoxycorticosterone acetate per day. When the dosage was increased to 10 mg of the steroid per day the glycosuria was exacerbated in all of the animals and decreased to the pre-injection level when the administration of the steroid was stopped.

⁴ Selye, H., and Dosne, C., *Endocrinology*, 1942, 30, 581.

TABLE I.
Influence of Temperature on Hemagglutination Titer.

Dilution	0*	2	4	8	16	32	64	128	Control
Sheep cells:									
0°C	+	+	+	sl+	0	0	0	0	0
45°C	0	0	0	0	0	0	0	0	0
Chicken cells:									
0°C	+	+	+	+	sl+	0	0	0	0
45°C	+	+	sl+	sl+	0	0	sl+	0	0

Each tube contained a mixture of 1 ml of diluted toxin and 1 ml of red cells. Saline was used as diluent. There were 131×10^6 cells per tube in the case of the sheep cells and 38×10^6 cells per tube in the case of the chicken cells. These numbers of cells were chosen so as to give about 3.45 times as many sheep cells as chicken cells which is about the reported difference in surface area of these cells. Agglutination was read by "pattern" test.

* This tube contained about 25,000 mouse MLD, so that as little as 1-3,000 MLD are giving macroscopic evidences of agglutination in the endpoint tubes.

The agglutination can be followed either microscopically or macroscopically. Methods in current use for observation of virus hemagglutination¹⁻³ are applicable to studies with the toxin. A most interesting difference from the experience with viruses is that the toxin does not appear to be taken up by the clumped red cells under the conditions studied: chicken and sheep cells at 0°C and room temperature; guinea pig cells at 30°C. The suspending menstruum suffers no reduction in toxic titre by mouse titration when agglutinated cells are centrifuged off. The centrifuged red cells themselves show a low (less than 99% of the toxin) and irregular order of toxicity which in separate tests has been reduced or abolished by washing with saline. The toxicity can be as logically attributed to the cells being contaminated with slight quantities of toxin by reason of the residual fluid wetting the cells after centrifugation as to a real adsorption phenomenon.

The addition of formaldehyde to toxin solutions results in diminished toxicity and hemagglutination. Other conditions that reduce the toxic titre such as aging in a dilute solution of salt, also decrease the hemagglutination titre. A corollary observation is that the employment of a peptone-saline mixture as diluent in place of the use of saline alone often gives an increased hemagglutination titre. Such a protective colloid effect is not unexpected in

view of the readiness with which purified toxin is biologically inactivated by surface denaturation. These observations make it highly unlikely that a non-toxic fraction can split off from the toxin molecule and cause hemagglutination independent of a toxic fraction which remains behind in solution.

The hemagglutination reaction is specifically prevented by type A antitoxin. Commercially prepared monovalent types A, B, and C horse antitoxins have been tested. The order of adding the reagents namely, toxin, antitoxin and red cells does not affect the inhibition by antitoxin. These observations provide conclusive proof that the hemagglutinating activity of the toxin preparations used was due to the toxin itself rather than to contaminating substances. Because ribonucleic acid is a major foreign material that accompanies the toxin fractions during several stages of purification, separate tests for hemagglutination by purified ribonucleic acid were performed. The ribonucleic acid was isolated from cultures of the same medium and strain of organisms as used for toxin production. Purified yeast nucleic acid was also tested. These nucleic acids did not agglutinate red cells.

The titre of a given mixture of toxin and red cells was found to vary with the temperature. But the influence of temperature was itself conditioned by the animal source of the red cells. Table I illustrates these findings. In attempting to compare red cells from different species, sheep and chicken cells were studied since among the common laboratory

¹ Hirst, G. K., *J. Exp. Med.*, 1942, 76, 195.

² Hirst, G. K., and Pickels, E. G., *J. Immunol.*, 1942, 45, 273.

³ Salk, J. E., *J. Immunol.*, 1944, 49, 57.

TABLE I.
Effect of Time and Temperature on Inhibition of Rat Brain Oxygen Consumption by Ethyl Alcohol. See text from experimental details. Oxygen consumption expressed as QO_2 for time periods given.

Temp. °C	Time after addition of alcohol, min.	Cone. of ethyl alcohol—% by volume					
		0	1.90	3.80	5.70	7.60	9.50
37.7	30- 60	11.02	11.74	10.82	9.89	8.24	3.70
	60-120	11.02	11.74	9.68	7.82	3.61	1.39
	120-180	11.02	11.74	8.14	4.58	1.03	1.03
30.0	30- 60	5.25	6.59	5.30	5.46	4.74	4.02
	60-120	5.25	6.08	5.30	5.46	4.17	2.68
	120-180	5.25	5.87	5.30	5.46	3.45	1.91
20.0	30-180 (Constant)	1.96	2.27	1.96	1.85	1.55	1.55

termed the "inhibitor stable" respiration. It has been found to be of approximately equal magnitude in brain slices maintained for over 3 hours without added substrate⁴ and in the presence of a number of other inhibitors.^{5,6-7}

It may be seen from Table I that low concentrations of alcohol appeared to produce an increase in the rate of oxygen consumption at all 3 temperatures. The effective concentration for this augmentation was about 2%. A similar increase in brain QO_2 with alcohol concentrations of from 1.6 to 3.2% has been

⁴ Fuhrman, F. A., and Field, J., 2nd., unpublished results.

⁵ Fuhrman, F. A., and Field, J., 2nd., *J. Cell. and Comp. Physiol.*, 1942, **19**, 351.

⁶ Fuhrman, F. A., and Field, J., 2nd., *J. Pharm. Exp. Therap.*, 1943, **77**, 392.

⁷ Fuhrman, F. A., *Abst. of Dissertations, Stanford University*, 1943, **19**, 3.

reported by Levy and Olszycka.⁸ It is possible that this increased rate of oxygen consumption is due to alcohol metabolism by brain tissue.⁹

Summary. The inhibition of oxygen consumption of rat cerebral cortex slices by ethyl alcohol was studied at 37.7°C, 30°C and 20° C. The inhibition becomes progressively greater with time at 37.7°C; such progressive inhibition is less marked at 30°C and is absent at 20°C. The minimum effective inhibitory concentration increases with decrease in temperature. The percentage inhibition with a given concentration of alcohol decreases with decrease in temperature.

⁸ Levy, J., and Olszycka, L., *Compt. rend. Soc. Biol.*, 1940, **133**, 370.

⁹ Himwich, H. E., Nahum, L. H., Rakieten, N., Fazekas, J. F., DuBois, D., and Gildea, E. F., *J.A.M.A.*, 1933, **100**, 651.

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. Hemagglutination by Botulinal Toxin.

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In the course of studies of the properties of type A botulinal toxin we have observed that the addition of toxin to red cell suspensions results in agglutination. Crystalline and amorphous preparations of toxin have proven to

be equally capable of causing hemagglutination. Observations for agglutination have been positive with red cells from all animal species tested (chicken, guinea pig, rabbit, sheep, man.)

TABLE IV.
Inability of Antitoxin to Redisperse Agglutinated Chicken Red Cells.

Dilution of toxin	0*	2	4	8	16	32	64	Control
Treatment								
Set 1: Toxin + 0.2 ml antitoxin for 30 min. at 25°C before adding red cells. Readings overnight at 10°C	+	0	0	0	0	0	0	0
Set 2: Toxin + 0.2 ml saline for 30 min. at 25°C before adding red cells. Reading overnight at 10°C	+	+	+	+	+	+	0	0
Set 2: Reshaken, 0.2 ml antitoxin added. Kept at 25°C 2 hr, then at 10°C overnight for agglutination reading.	+	+	+	+	sl+	0	0	0

Horse antitoxin used was commercially prepared. According to the manufacturer's statement of potency about 3 units of antitoxin would be present in the 0.2 ml added to each tube of the experimental set. Red cells per tube were 40×10^6 . Agglutination was read by "pattern" on bottom of tube.

* 100,000 mouse MLD of toxin were present in the first tubes.

had been agglutinated at 0°C when washed at 45°C showed a complete loss of agglutination. A duplicate set washed at 5°C showed persistence of agglutination in the tubes originally containing the greatest amounts of toxin. There was only a one or two tube reduction in endpoint with the 2-fold dilution schedule employed.

Agglutinated sheep cells washed free of toxin at 45°C so that they were no longer agglutinable at 0°C remained capable of being reagglutinated by the addition of fresh toxin.

As already stated antitoxin was found to inhibit hemagglutination irrespective of the order in which the reagents were mixed initially. But it was of some interest to learn whether or not the addition of antitoxin could disperse cells which had been once agglutinated. Accordingly experiments to test this point were performed. The antitoxin proved relatively incapable of dispersing agglutinated chicken red cells. Typical data are given in Table IV. In still other experiments the agglutinated chicken cells remained agglutinated even when washed free of toxin with saline, and then resuspended in a quantity of antitoxin sufficient to inhibit agglutination if the reagents had been added together at the beginning of the trial. Experiments along similar lines with sheep cells were performed but are not recorded since results were irregular and inconclusive.

The observations discussed are preliminary to an effort to explain hemagglutination by the toxin, and to learn whether hemagglutination can be employed as a quantitative meas-

ure of toxin. They have been presented for the purpose of bringing the phenomenon into common knowledge. It is not possible at the moment to finally decide whether the agglutination is due to an enzymatic action by the toxin on the red cell, or to some purely physical effect of the toxin solutions that unsettles the balance of cohesive and repulsive forces normally operative at the red cell surface. Nor is it possible to conclude whether one type of mechanism is acting in the case of chicken cells and another for the sheep cells. But an additional explanation of the results exists which is probably not supported by the evidence at hand. Lamanna and Doak⁴ have found that normal serum can be precipitated by botulinal toxin. If in the studies performed a contaminating layer of normal serum proteins was present at the cell's surface then the agglutination of the red cells might be more apparent than real. The reaction between toxin and the serum might well explain the results. Objections to the occurrence of this event are as follows: All the chicken and sheep red cells used were washed by centrifugation from saline or a buffer-saline mixture. The amount of residual serum proteins must be minimal. Guinea pig red cells when washed four times by centrifugation from mammalian Ringer's solution remained agglutinable by toxin. The addition of normal serum to a mixture of red cells and toxin did not affect the agglutination titre.

⁴ Lamanna, C., and Doak, B. W., *J. Immunol.*, 1948, 59, 231.

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TABLE II.
Relative Lack of Dependence of Hemagglutination Titer at a Given Temperature upon Prior Exposure at Another Temperature.

Dilution	0	2	4	8	16	32	64	128	256	512	Control
Temp. of exposure °C											
0	+	+	+	+	+	+	+	+	0	0	0
Room	+	+	sl+	sl+	0	0	0	0	0	0	0
39	0	0	0	0	0	0	0	0			0
45	0	0	0	0	0	0	0	0			0
Above tubes reshaken and placed at different temperatures:											
45°C set at 0°C	+	+	+	+	+	+	+	sl+			0
39°C set at room	+	+	sl+	0	0	0	0	0			0
Room set at 39°C	0	0	0	0	0	0	0	0			0
0°C set at 45°C	0	0	0	0	0	0	0	0	0	0	0

Sheep cells used. Agglutination read by "pattern" test.

TABLE III.
Effect of Washing Upon Agglutinated Red Cells.
Chicken cells: 38×10^6 per tube.

Dilution	Temperature of		0*	2	4	8	16	32	Control
First agglutination, °C	Washing, °C	Agglutination after washing, °C	Agglutination results						
10	—	—	+	+	+	+	sl+	0	0
45	45	10	+	+	+	+	hemolyzed	hemolyzed	0
	25	45	+	+	sl+	sl+	0	0	0
			+	+	+	+	0	0	0
Sheep cells: 131×10^6 per tube.									
10	45	10	+	+	+	sl+	0	0	0
45	—	—	0	0	0	0	0	0	0
	25	10	0	0	0	0	0	0	0
45	—	—	0	0	0	0	0	0	0
	kept at 45° then placed at 10°		0	0	0	0	0	0	0
			+	+	sl+	0	0	0	0

* Tube contains 25,000 mouse MLD of crystalline toxin per ml.

Results within each horizontal column are those obtained with a single set of tubes. A total volume of 2 ml was present per tube. Diluent and wash fluid was saline. Washing was done twice by centrifugation of the cells from 5 ml of fresh saline at the indicated temperature.

animals they represent cell types with extensive morphological differences in both structure and size. It was thought to be better to use concentrations of these cells with equivalent surface areas rather than equal numbers since the effects of the toxin are assumed to primarily involve surface phenomena or structures. The sheep cells were more sensitive than the chicken cells to the temperature variable. But with both types of cells the effect of temperature was found to be relatively independent of the previous temperature exposures of red cell-toxin mixtures. Data for 0°C about the same endpoint is recorded sheep cells given in Table II show that at whether the test is conducted initially at 0°C

or the cell-toxin mixture is initially held at 45°C and then placed at 0°C. Exposures at the other 3 temperatures listed in Table II show the same characteristic.

An interesting difference between agglutinated sheep and chicken cells was in their behavior when washed free of the suspending toxic fluid (Table III). Once chicken cells have been agglutinated they tend to remain agglutinated irrespective of washing or the temperature of washing. With agglutinated sheep cells the effect of washing was more complex. Washing tended to reduce the persistence of agglutination but was conditioned by the temperature of washing. In one experiment a set of tubes of sheep cells which

TABLE I.
Effect of Skin Extracts and Control Solutions on Mammary Tumors in Mice.

Case No.	Animal No.	Extract or control solution	Dosage (cc)	No. of injections	Original size of tumor* (mm)	Size 2 days after last injection† (mm)
1	Cn 1	Cntr.	0.3	6	34	33/12
2	Cn 2	Cntr.	0.3	6	37	22/12
3	Cn 3	Cntr.	0.3	6	40	29/12
4	CF 1	CS	0.3	6	29	0/12
5	CF 2	CS	0.3	6	33	4/12
6	CF 3	CS	0.3	6	31	7/12
7	CF 4	CS	0.3	6	30	0/12
8	Cn 4	Cntr.	0.15	6	26	24/12
9	Cn 5	Cntr.	0.15	6	24	14/12
10	Cn 6	Cntr.	0.15	6	25	16/12
11	CF 5	CS	0.15	6	22	7/12
12	CF 6	CS	0.15	6	17	0/12
13	CF 7	CS	0.15	6	24	0/12
14	Cn 7	Cntr.	0.05	9	30	32/18
15	Cn 8	Cntr.	0.05	9	21	17/18
16	CF 8	CS	0.05	9	20	0/18
17	CF 9	CS	0.05	9	29	14/18
18	CF 10	CS	0.05	9	31	11/18
Subcutaneous Injections.						
19	Cn 9	Cntr.	0.3	9	35	40/18
20	CF 11	CS	0.15 and 0.3‡	9	25	22/18
21	CF 12	CS	0.15 and 0.3‡	9	29	22/18
22	CF 13	CS	0.3	9	40	38/18

* Length plus breadth.

† The numerator = length plus breadth; denominator = number of days since first injection.

‡ Five injections of 0.15 cc followed by four of 0.3 cc.

the effects of extracts of degenerating amphibian tail muscle and skin on spontaneous tumors in mice.

Materials and methods. Extracts of normal and degenerating anuran larval tail muscle and skin were prepared. Preliminary tests showed that those of degenerating skin were most effective. These (Extract CS) were prepared as follows: *R. catesbeiana* larvae were selected in a metamorphic stage at which tail resorption was well underway. The tail integuments of approximately 60 larvae were stripped off and ground to a homogeneous consistency in a mortar. 25 cc of cold 5N HCl was added to 5 cc of the ground skin and the mixture allowed to stand, at room temperature, for 48 hours. The mixture was then filtered several times and the final filtrate neutralized against litmus with dry NaHCO_3 . A small amount of sterile distilled water was now added to bring the volume up to 25 cc. The extract was then placed in a sterile bottle and refrigerated until ready to use. Neutral control solutions, for

injection, were also prepared with 5N HCl and NaHCO_3 .

CFL strain mice* of uniform age and showing definite surface indications of early spontaneous mammary tumor growth were utilized for injection purposes. The mice selected for injection were placed in individual cages and maintained under uniform diet conditions for at least a week prior to use in the tests. The selection of individuals for extract and control solution injection purposes was made on the basis of general healthiness of the animals and similarity in tumor size and location. Extracts and control solutions were injected in most cases directly into the tumors and in a few cases subcutaneously on the back. The injections were made every other day.

Results. The results are listed in Table I. They include only those animals which lived for more than 10 days following the last injection. Such mortality (27%) as occurred was

* The mice were supplied by Carworth Farms, New City, N. Y.

Yet the large excess of serum in these cases might logically be expected to compete for the toxin and thus reduce the hemagglutination titre. If normal serum proteins were in some way "bound" at the red cell's surface antitoxin might be similarly bound. The addition of toxin to a red cell-antitoxin mixture should then be expected to result in agglutination. Actually a mixture of sheep cells and horse antitoxin incubated for an hour before addition of toxin remains unagglutinated. Finally, the presence of serum at the cell surface should show some, if slight, toxin adsorption if such serum were actually responsible for agglutination. An experiment was performed in which 460 millions of sheep red cells were mixed with only 200 mouse MLD of toxin at 10°C. Putnam *et al.*⁵ have determined that the number of molecules per LD₅₀ is of the order of 2.1×10^7 . An MLD would

⁵ Putnam, F. W., Lamanna, C., and Sharp, D. G., *J. Biol. Chem.*, 1946, **165**, 735.

contain less than twice as many molecules.⁶ Thus in the mixture used there was only of the order of 18 toxin molecules per cell. The adsorption of only a few molecules per red cell would have resulted in a large percentage adsorption of toxin. Yet when the red cells were centrifuged down and the supernate titrated no significant reduction of toxic titre was found.

Summary. Type A botulinal toxin causes agglutination of chicken, guinea pig, rabbit, sheep and human red cells. The agglutination is not accompanied by evidence of adsorption of the toxin by the red cells. Temperature, particularly in the case of sheep cells, influences the hemagglutination titre. When washed free of toxin, agglutinated chicken cells tend to remain agglutinated while agglutinated sheep cells are more readily dispersed.

⁶ Lamanna, C., McElroy, O. E., and Eklund, H. W., *Science*, 1946, **103**, 613.

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Regression and Reabsorption of Mammary Tumors by Extracts of Degenerating Amphibian Skin.

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The chemotherapy of cancer has received and still receives considerable attention. Most work has been directed towards the examination of substances which inhibit growth as contrasted with those which are curative in the sense of bringing about the complete regression of the tumor. The works of Roffo¹ and of Boyland² were of especial interest to the writer and led to the experiments here described. Roffo first found that the injection of fresh extracts of striated muscle into rats implanted with a transferable fibro-sarcoma 10-19 days previously were without inhibitory or regressional effects. The same

negative findings were recorded when tumorous tissue was tested *in vitro*. However, striking regression and disappearance of tumorous tissue was obtained when applying hydrolysates of striated muscles both *in vivo* and *in vitro*. Boyland was able to demonstrate less striking results on grafted sarcomata and spontaneous carcinomata in mice following oral administrations of acid extracts of ox heart muscle. Pronounced inhibition of tumor growth was obtained but no cases of complete regression.

Reasoning from a general hypothesis that degenerating tissues might, in certain cases, contain a substance or substances detrimental to the growth or persistence of tumorous tissue, experiments were planned involving

¹ Roffo, A. H., *Biol. Inst. Med. Exp.* (Buenos Aires), 1938, **14**, 257.

² Boyland, Eric, *Biochem. J.*, 1941, **35**, 1283.

Response of Normal and Malignant Lymphoid Tissue to Non Specific Tissue Damage.*

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In the evaluation of possible therapeutic agents in experimental lymphoma it has become apparent that drugs producing lymphoid tissue atrophy may do so either by direct action on the lymphocyte itself or an indirect action initiating an "alarm" reaction. Bass and Freeman¹ using 95% ethyl alcohol as a stimulus have shown that lymphoma 6C3HED responds in a manner similar to that of normal lymphoid tissue. That the alcohol itself was not a lympholytic agent was evident, as larger amounts of ethyl alcohol diluted to 19% concentration produced no lympholytic effects. Certain agents, such as nitrogen mustard and urethane, produce atrophy of lymphoid tissue through two possible mechanisms: (a) initiation of an alarm reaction^{2,3} and (b) a direct action of the drug upon the lymphoid organs. The latter effect, which is the most important one, has been demonstrated by administration of these agents to adrenalectomized mice bearing lymphoid tumors.⁴ It is, therefore, apparent that screening technics for new lympholytic agents must take into consideration both the direct and indirect effect upon lymphoid tissue. The present study was undertaken to establish further relationship of malignant tissue response to the administration of established stress stimuli. Those selected for the study

were 40% glucose⁵ and 4% formaldehyde.⁶ Since it has been reported by Selye,⁷ using the rat as his experimental animal, that fasting is either an alarming stimulus in itself or potentiates alarming stimuli, the effect of fasting in mice was also included in this study.

Materials and methods. The pure strain C3H male mice employed in this study were obtained from Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine. The C3H F₁ hybrids of mixed sexes were bred in this laboratory. All animals were maintained on Purina dog chow and allowed free access to water at all times.

Transplanted lymphoma, 6C3HED, was employed in this investigation. A small particle of tumor tissue was implanted subcutaneously into the right axillary region of each animal by means of a 15 gauge needle. Procedures initiating alarming stimuli were instituted when tumors had become definitely measurable (seven days after implantation in pure strain C3H mice; seven to eleven days after implantation in C3H F₁ hybrid mice).

Intraperitoneal injections of 95% ethyl alcohol, U. S. P., were given to tumor animals in daily doses of 0.02 ml per mouse for 2 days. Animals used for spleen and thymus weight studies received 1.5 ml per kg at the same intervals. An aqueous solution of 40% glucose was administered intraperitoneally in doses of 150 ml per kg of body weight daily for 2 days. Subcutaneous injections of 4% formaldehyde were given twice daily for two days in doses of 5 ml per kg of body weight. Agents repeated within one day were given

* This investigation was supported by a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

¹ Bass, A. D., and Freeman, M. L. H., *Science*, 1948, **107**, 114.

² Ludewig, S., and Chanutin, A., *Endocrinol.*, 1946, **38**, 376.

³ Karnofsky, D., Graef, I., and Smith, H. W., *Am. J. Path.*, 1948, **24**, 275.

⁴ Bass, A. D., and Feigelson, M., *Cancer Research*, in press.

⁵ Reiss, M., Macleod, L., Golla, Y., *J. Endocrinol.*, 1943, **3**, 292.

⁶ Selye, H., *Brit. J. Exp. Path.*, 1936, **17**, 234.

⁷ Selye, H., *J. Clin. Endocrinol.*, 1946, **6**, 117.

distributed quite equally among extract and control injected and non-injected stock animals. The mortality was attributed entirely to extreme fluctuations in room temperature beyond control of the writer. The injected animals lived frequently for 30 or more days following the last injection. In certain individuals no new tumors appeared. In others one or two developed but never in close proximity to the injection site. For most cases involving tumor regression the effect was noted within 3 to 6 days, after which reduction in size progressed steadily.

In 6 cases (not listed in Table I) injections were terminated when the tumors were reduced approximately 50%. In 4 of these, tumor regression continued, but at a lowered rate, while in the other 2 regression ceased shortly and the tumors enlarged subsequently. New tumors appeared in several of the cases listed in Table I at the same time the injected tumors were undergoing reduction in size. This was not true, however, of any of the subcutaneously injected animals.

A study of the results listed in Table I will indicate that tumors showed definite regression following injections of control solutions in doses of 0.3 and 0.15 cc while 0.05 cc injections were relatively ineffective. Extract injected tumors, however, evidenced much greater regression in rate and size in all dosages used and accounted for the five cases of complete resorption.

Histological studies were made at successive growth stages of normally developing CFI strain mammary tumors and also of tumors undergoing degeneration as the result of control solution and skin extract injections. The normal stock tumors showed the usual transformations from simple adenomatous to malignant carcinomatous types. Tumors in-

jected several times with control solutions usually showed cystic development localized in one or more regions of the growth with large masses of carcinomatous tissue and some adenomatous in the remaining regions. In contrast, extract injected tumors after several injections evidenced larger and more numerous cysts accompanied by considerable amounts of adenomatous but very little carcinomatous tissue.

The subcutaneous injection of skin extract and control solution (Table I, cases 19-22) is of interest in that inhibition of tumor growth and slight regression in size is suggested. The number of cases is too small, however, to justify any definite conclusion.

Summary. Neutralized acid extracts of degenerating tail skin from involuting *R. catesbeiana* larvae were injected into rapidly growing spontaneous mammary tumors in mice. Marked regression of the tumors was obtained in all instances resulting in complete disappearance of all tumorous tissue in 5 out of 13 cases within eight to eighteen days after the initial injection.

The application of neutralized acid control solutions, similarly injected, resulted with one exception in slight to marked reduction in tumor size but never in complete resorption.

The tumors of one control and 3 extract animals, subcutaneously injected, showed only slight regression in size but are indicative in that growth was apparently inhibited.

The results indicate that a neutralized HCl acid extract of degenerating anuran tail skin contains some substance or substances possessing inhibitory and degenerative effects on spontaneous mammary tumors in mice beyond those exhibited by a neutralized acid control solution similarly injected.

TABLE I.
C3H Hybrids (Mixed Sexes) Sacrificed 48 Hours After Initial Therapy.

Treatment	Dose schedule	No. animals	Organ weights, g per 100 g body wt	
			Mean spleen wt \pm S.E.	Mean thymus wt \pm S.E.
None	—	15	1.348 \pm 0.0802	0.153 \pm 0.0194
95% ethyl alcohol	1.5 ml/kg daily for 2 days	7	1.050 \pm 0.1259	0.051 \pm 0.0101*
4% formaldehyde	5 ml/kg 2 times daily for 2 days	9	0.605 \pm 0.742*	0.050 \pm 0.0056*
40% glucose	15 ml/kg daily for 2 days	10	1.055 \pm 0.1187	0.112 \pm 0.0152
48-hour fast	—	9	1.138 \pm 0.1031	0.107 \pm 0.0147*

* Upon statistical analysis weights are significantly smaller than untreated controls.

temporarily inhibit growth or produce temporary regression of the malignant lymphoid tumor 6C3HED. A similar effect is produced by a 48-hour fast. Fasting begun 30 hours after the initial stimulus gives an additional "lympholytic" effect.

It has been shown that stimuli which are known to produce an alarm reaction with resulting atrophy of the normal thymus and normal spleen produce a similar type of reaction in tissue composed of malignant lymphocytes.

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Human Skin Grafted Upon the Chorioallantois of the Chick Embryo for Virus Cultivation.*

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(Introduced by Werner Henle.)

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Goodpasture, Anderson, and Douglas have described a technic for grafting human skin onto the chorioallantoic membrane of embryonated eggs.^{1,2} They demonstrated that viruses such as herpes simplex and vaccinia, which multiply readily in the normal embryonated egg, would also grow in the grafted skin with the appearance of characteristic inclusion bodies in the epithelial cells. In addition, by using this technic they recorded an experiment in which they were able to grow herpes

zoster on a single occasion.³ They also indicated the value of such heterologous skin grafting in immunologic studies, by demonstrating that skin taken from chickens immune to fowl pox would lose its immunity after being grafted onto the chorioallantois.⁴ It is assumed that the immune bodies are diluted out of the skin as it grows in the egg.

The obvious usefulness of vascularized, morphologically distinct human skin, actively growing in a sterile host that does not produce antibodies of its own, prompted the

* This study was supported by grants from The Lederle Laboratories Division, American Cyanamid Company, the U. S. Public Health Service, and the National Foundation for Infantile Paralysis.

[†] Fellow in the Medical Sciences, National Research Council, during part of this study.

[‡] Senior Fellow in Virus Research, National Research Council.

¹ Goodpasture, E. W., Douglas, B., and Anderson, K., *J. Exp. Med.*, 1938, **68**, 891.

² Goodpasture, E. W., and Anderson, K., *Am. J. Path.*, 1942, **18**, 563.

³ Goodpasture, E. W., and Anderson, K., *Am. J. Path.*, 1944, **20**, 447.

⁴ Goodpasture, E. W., and Anderson, K., *Arch. Path.*, 1940, **30**, 212.

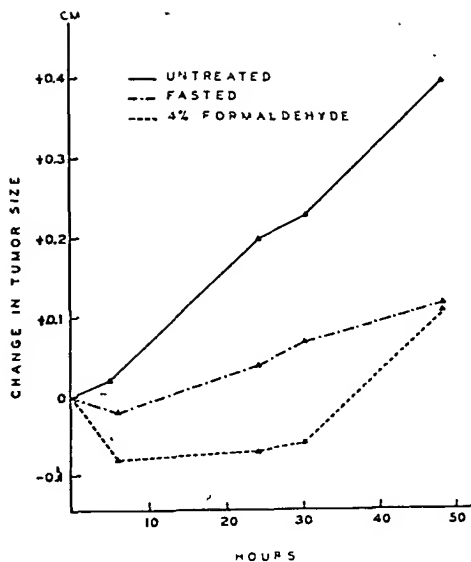


FIG. 1.

C3H hybrid mice were used. Number of animals employed: untreated 6, fasted 9, formaldehyde 10. Therapy was started at zero time.

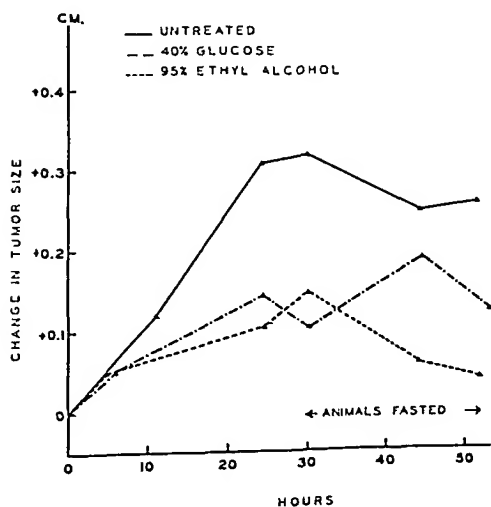


FIG. 2.

C3H mice were used. Number of animals employed: untreated 11, 95% alcohol 11, 40% glucose 12. Treatment was begun at zero time.

6 hours apart. Fasted animals were deprived of all food but were given water *ad libitum*. Tumor measurements were made twice daily. The longitudinal and transverse diameters of each tumor were measured with calipers and the mean dimension (one half of the length plus the width expressed in centimeters) was

arbitrarily used as the tumor size. Animals used for organ weight studies were killed with ether 48 hours after therapy was initiated. Spleens and thymuses were carefully dissected out and weighed immediately on a Roller-Smith micro torsion balance.

Results. The effect of subcutaneous injection of 4% formaldehyde and of a 48 hour fast upon the tumor growth in C3H hybrid mice of mixed sexes is illustrated in Fig. 1. It is clearly shown that the tumor growth curve of these treated animals is definitely altered from that of the untreated animals. Formaldehyde administration induced a definite regression whereas fasting induced only an inhibition of tumor growth. Fig. 2 shows the effect of two other alarm stimuli (95% ethyl alcohol administered intraperitoneally and 40% glucose administered intraperitoneally) on the change in tumor size in pure strain C3H male mice. Beginning 30 hours after the initial stimuli all animals were fasted for a period of 24 hours. Both stimuli (ethyl alcohol and glucose) are shown to inhibit tumor growth during the initial 30 hour period. Fasting under these conditions definitely potentiated the inhibiting effects of 95% ethyl alcohol and resulted in tumor regression in untreated animals. A less definite effect on the glucose treated animals was observed.

Table I demonstrates the effect of 95% ethyl alcohol, 4% formaldehyde, 40% glucose and a 48-hour fast on splenic and thymic wet weights in C3H F₁ hybrid mice of mixed sexes. Formaldehyde produced a statistically significant reduction in both spleen and thymus weights. Thymus weights of ethyl alcohol-treated animals and of fasted animals were significantly less than those of the untreated controls. Although all of the means of organ weights were not statistically significant, the mean spleen and thymus weights of all treated animals were lower than the untreated controls.

Summary. Further evidence has been added to indicate that ethyl alcohol is an alarming stimulus. We have shown that the non-specific stimuli, 4% formaldehyde and 40% glucose, in unadrenalectomized mice



FIG. 3.

Epithelium of grafted human skin infected with herpes simplex 48 hours before fixation. This skin has been growing for 27 days (in 4 eggs) before inoculation with the virus. Intranuclear inclusion bodies in various stages of development are present. (H & E $\times 700$).

leaving the epidermis and attached corium as thin as possible. Throughout this procedure, the antibiotic gelatin saline solution was used generously to prevent the tissue from drying. The thin sheet of skin was then cut into squares of the desired size. Ten-day-old embryonated hens' eggs were prepared by the "false air sac technic,"⁶ and a window was made in the side of the egg overlying the false air sac by removing pieces of shell with the aid of fine forceps. A sterile, flat instrument, such as a forceps handle, was used to place the skin on the chorioallantoic membrane of the eggs, with the raw surface in even contact with the membrane. After grafting the skin, the shell defect was sealed with 2 layers of Scotch tape, and the eggs were incubated on their sides at 96°F.

The grafted skin was inoculated with virus-containing material 24 hours later, under the assumption that humoral antibodies from the donor of the skin would be largely removed after this interval on the egg.² The grafted skin was inoculated through a window cut in the Scotch tape with sterile scissors, after preliminary cleansing with 70% alcohol.

Material to be inoculated was placed directly on the skin, which was then pricked with a vaccinating needle or with a 27-gauge hypodermic needle of the syringe used to deposit the inoculum onto the skin. More Scotch tape was used to seal the window again, and the eggs incubated for an additional period, varying from 1 to 7 days as the particular experiment required.

At the appropriate time, the skin was removed with attached chorioallantoic membrane. Material for histological study was placed on a small piece of absorbent paper with the membrane next to the paper, and the whole fixed in 5% acetic acid Zenker's fixative. Hematoxylin- and eosin-stained paraffin sections were prepared for routine study.

For passage to another egg, the skin was removed aseptically and placed in a sterile petri dish. With the aid of iris scissors and forceps, all of the attached chorioallantois was trimmed away, as was any additional connective tissue which had grown in the corium during the initial period on the egg. The skin was then transferred to the chorioallantois of another 10-day egg, prepared in the usual fashion. Such serial passages were usually made at 7-day intervals.

⁶ American Public Health Association, Diagnostic Procedures for Virus and Rickettsial Diseases, p. 243, 1948.

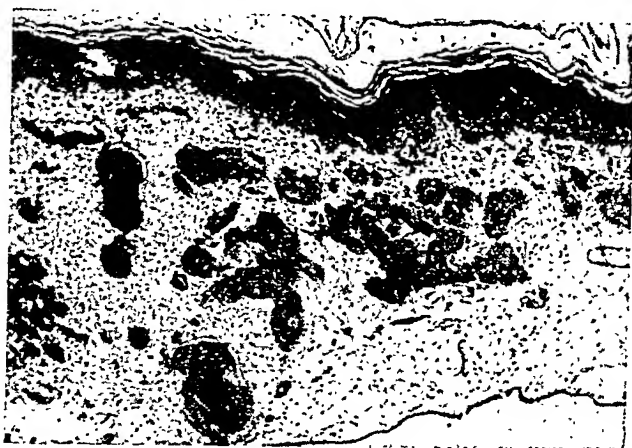


Fig. 1.

Human skin, 7 days after grafting onto chorioallantois. Large blood vessels contain nucleated erythrocytes, and are prominent in the denser human corium as well as the chick membrane. (H & E $\times 40$).

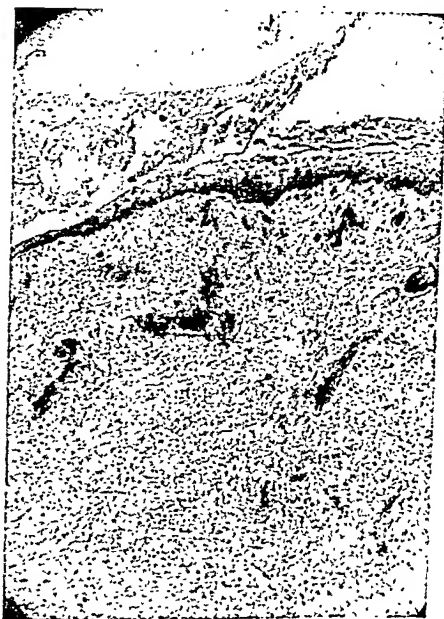


Fig. 2.

Human skin, after grafting onto 3 successive eggs for a total of 21 days. The epidermis remains distinctive. The dense collagen bundles of the corium have been separated by inflammatory cells of the chick. (H & E $\times 40$).

study herein reported. This was to try to develop a relatively simple procedure whereby bacteria-free, growing skin was available

which could be kept growing by serial transfer from egg to egg.

Technic. The human skin used in most of this study was obtained at circumcision of white and Negro children.[§] The prepuce was cleaned preoperatively with soap and water and alcohol. Other antiseptics were avoided. The skin was used routinely within 2 hours of operation, but occasionally was stored in the ice box at 4°C for as long as 5 days before grafting. The prepuce was further cleaned of clotted blood and debris in the laboratory by bathing with the following solution: Physiological saline solution, phosphate buffered to pH 7.2, to which had been added gelatin to make 0.5% solution, and penicillin and streptomycin to make a final concentration of 500 units and 100 μg per ml, respectively. Previous studies in this laboratory⁷ have indicated the antibacterial effectiveness and innocuous character of this solution. The skin was stretched, with sterile pins, on a sterile, solid cork board, epithelial surface down. With forceps and scalpel, the connective tissue was meticulously removed,

[§] Obtained through the courtesy of Dr. C. E. Koop and the Surgical Staff of the Children's Hospital of Philadelphia.

⁷ Coriell, L. L., Blauk, H., and Scott, T. F. McNair, to be published.

tional proof of the viability of the growing epidermis (Fig. 3). If the skin is inoculated with vaccinia virus, characteristic cytoplasmic basophilic inclusion bodies are formed in the epithelial cells (Fig. 4).

In addition, we were able to grow the virus of herpes zoster on one occasion (Fig. 5). This was done by inoculating skin which had been growing on the chorioallantois for 24 hours. The inoculum was untreated vesicle fluid taken from a patient with herpes zoster ophthalmicus of 48 hours' duration. The skin was removed after 6 days' additional incubation. The microscopic appearance of the infected area, including the presence of a large number of characteristic intranuclear inclusion bodies, confirms Goodpasture's observation in his single successful cultivation

of the zoster virus.³

Summary and Conclusions. (1) Human skin may readily be grafted onto the chorioallantoic membrane of embryonated eggs. (2) The prepuce removed by usual surgical circumcision is a satisfactory source of adequate amounts of skin. (3) Penicillin and streptomycin obviate most bacterial contamination. (4) Grafted human skin may be passed serially from egg to egg at weekly intervals and remain viable. (5) Herpes simplex and vaccinia grow readily on grafted human skin, with the formation of characteristic inclusion bodies. (6) A successful inoculation with herpes zoster confirms the previous single successful cultivation of this virus outside of the human body.

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Atrio-Ventricular Anastomosis: An Additional Valve.

A. RAPPAPOORT, J. F. MURRAY AND L. S. DAVIES. (Introduced by C. H. Best.)

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In vascular surgery during the last few years communications have been established experimentally and therapeutically between blood vessels. Little has been done in surgery of the heart involving the formation of anastomosis between the cavities of this organ. Schepelmann¹ and Dimitrieff² were the first to create artificial communications between the atria or the ventricles. Neither of these workers tried to produce a new opening that would join an atrium to a ventricle.

If such an anastomosis could be created with valvular properties it would be of value in pathological changes of the valves. In stenosis of the mitral valve, for example, the blood which has been dammed back in the atrium could stream through the new pathway into the left ventricle. In insufficiency,

on the other hand, the anastomosis could serve as a safety valve guarding against the rising pressure in the atrium. These points, however, remain to be settled experimentally.

Dimitrieff², Cutler³ and Souttar⁴ have shown that various operations on the auricular appendage are well tolerated. The appendage also represents the shortest anatomical pathway for producing a connection between atrium and ventricle. Furthermore, by using it for the atrio-ventricular anastomosis, valvular requirements can be fulfilled. Because of its funnel shaped structure, it is most suitable for transformation into a valve and for implantation into the ventricle. In an anastomosis using the appendage, the blood will flow from a broad base to a narrow apex, whereas back flow through this structure would be difficult. The experiments to be described

¹ Schepelmann, E., *Deutsche Z. f. Chir.*, 1912, 120, 562.

² Dimitrieff, I. P., *Zentralbl. f. Chir.*, 1926, 53, 715.

³ Cutler, E. C., and Beck, C. S., *Arch. Surg.*, 1929, 18, 403.

⁴ Souttar, H. W., *Brit. Med. J.*, 1925, 2, 603.

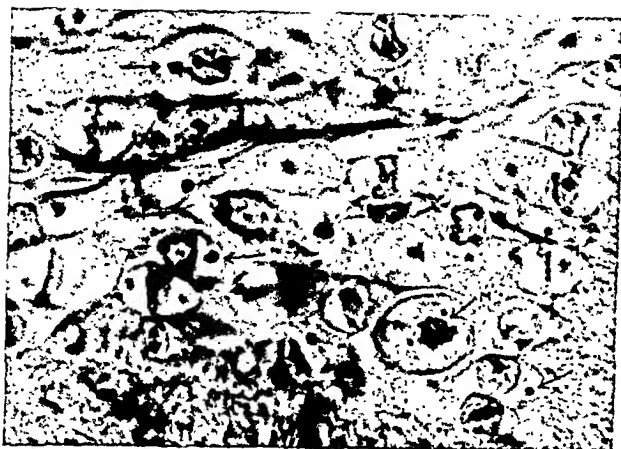


FIG. 4.

Epithelium of grafted human skin infected with vaccinia 72 hours before fixation. Numerous basophilic cytoplasmic inclusion bodies (Guarnieri) are present. A mitotic figure (M) can also be seen. (H & E $\times 450$).

Results. The observations of Goodpasture and his group that human skin would grow quite readily on the chick chorioallantois were confirmed. Of 245 grafts only 13 were unsuccessful. Bacterial contamination was rare in our experience, although fungi such as *aspergillus* or *candida* occasionally grew out. A successful "take" was accomplished by active proliferation of the epidermis and the establishment of chick circulation in the human corium, as indicated by nucleated chick erythrocytes in the blood vessels of the corium (Fig. 1). This epidermal proliferation produced an increasingly thick, horny layer, which was desquamated after 2 or 3 weeks of continuous passage. If the epithelium failed to grow, the histologic picture was quite distinct. The epithelial cells assumed a light pink color with a small, pyknotic, dense nucleus, and there was none of the usual evidence of multiplication such as mitotic figures or increase in thickness. It was further demonstrated that the human skin could be passed serially at weekly intervals from egg to egg, and retain distinctive epithelium (Fig. 2). Of 124 regrafts, 7 failed to take.



FIG. 5.

Epithelium of grafted human skin infected with herpes zoster 6 days before fixation. Almost every nucleus shows margined chromatin and contains a characteristic inclusion body. (H & E $\times 450$).

By inoculating skin which had thus been kept alive for 4 weeks (27 days) with herpes simplex virus, and incubating for an additional 48 hours, it was possible to demon-

strate the formation of characteristic intranuclear inclusion bodies in the epithelial cells of the skin graft. This was considered addi-

be the "guide thread" (Fig. 1). The ends of this thread are held in a clamp.

A suture is placed at the proximal limit of the opening on each side, tied into place (Fig. 1) and both free ends are threaded through a No. 6 curved round needle. They will be "temporary hemostatic sutures." The needles are clamped in needle holders and stuck in the drape sheets, ready for use.

Opening the Ventricle. A transverse incision is made in the anterior ventricular wall 5 mm below the posterior row of sutures already placed (Fig. 1). A broad scalpel or a punch which will produce a hole 15 mm long and 5 mm wide is used. After this has been pushed through the ventricular muscle a fine, curved Reverdin needle is introduced into the heart cavity about 3 cm below the ventricle opening. When it contacts the scalpel or punch the latter is pulled out, the Reverdin needle follows in its wake and the tip appears in the incision. The blood then spurts. The "guide thread" is threaded in the Reverdin needle and the latter is pulled out, quickly drawing the appendage into the incision and ventricular cavity. Hemorrhage is thereby diminished. While an assistant arrests further hemorrhage with direct digital pressure the "temporary hemostatic sutures", held in readiness, are guided into the ventricular cavity at the right and left corners of the incision. These are directed from within the cavity through the entire thickness of the ventricular wall (Fig. 2). Hemorrhage is

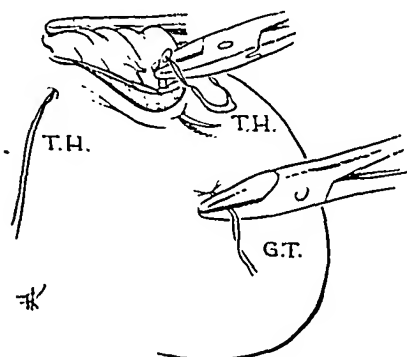


FIG. 2. Implantation of Auricular Appendage. Placing the "temporary hemostatic sutures." The auricular appendage is being held in position by a forceps on the "guide thread."

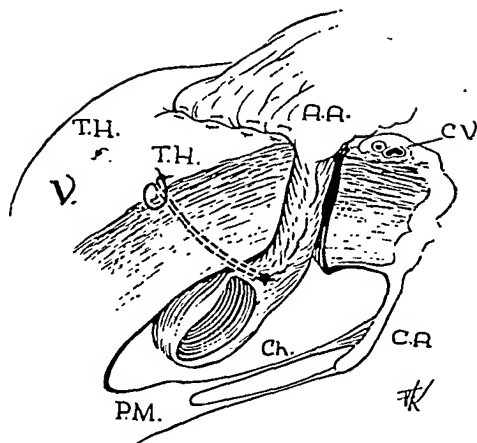


FIG. 3. Longitudinal Section of Anterior Ventricular Wall. Embedded Appendage "in situ." C.A. = Anterior cusp. Ch. = Chordae tendineae. P.M. = Papillary muscle. C.V. = Coronary vessels. T.H. = "Temporary hemostatic sutures"—anchored.

completely arrested when these are drawn together and held by a single knot fixed in a hemostat.

The anastomosis is completed by interrupted mattress sutures uniting the anterior wall of the appendage to the edge of the ventricular incision. The single knot of the "temporary hemostatic suture" is opened and each double thread is permanently fixed where it emerges. These anchor the edges of the auricular appendage to the endocardial margin of the incision and hold it deeply implanted in the ventricular cavity (Fig. 3). The guide thread is pulled out after cutting one end short. The auricular appendage, now mobile, lies between the chordae tendineae of the anterior valve and the anterior ventricular wall (Fig. 3). If the appendage is too short to be implanted into the ventricle, it can be embedded in an incision made closer to its base, behind the coronary vessels. These are retracted after careful dissection. The ventricle is opened by an obliquely placed incision on the upper limit of the ventricular myocardium, just below the A-V ring. The appendage is then also lodged between the chordae tendineae and anterior wall of the ventricle.

The pericardium is left open, 100,000 units

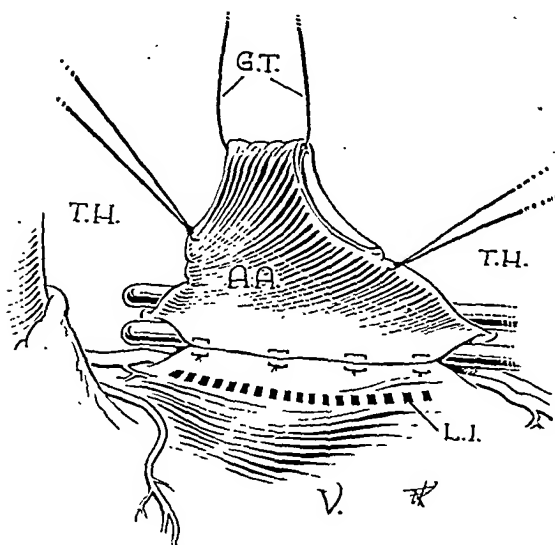


FIG. 1. Preparation of Auricular Appendage Showing Posterior Row of Sutures.

A.A. = Auricular appendage, posterior surface.

G.T. = "Guide thread."

L.I. = Line of ventricular incision.

T.H. = "Temporary hemostatic sutures."

V. = Ventricle.

were planned and initiated by the senior author (A.R.).

Technic. Dogs were anesthetized with intravenous nembutal and artificial respiration administered by positive pressure through an endotracheal tube. The animal was placed on the right side, a small pad under the right thorax providing the best exposure. The incision was usually made in the fourth intercostal space for the left auricle, the third intercostal space for the right. When the pleural cavity is opened a tampon soaked in 5% cocaine solution is used to paint the pericardium in order to block reflexes that might arise and disturb cardiac activity. Exposure is maintained by self-retaining rib spreaders and towels packing off the lungs. A longitudinal incision is made in the pericardium between two holding sutures which stretch and maintain the opening. The incision is carried up to the superior reflection of the pericardium. Cocaine solution (4%) is also introduced into the pericardial cavity and is spread over the epicardium by the heart contractions. The auricular appendage is gently grasped at its apex with tongue forceps,

and a narrow slightly curved clamp is fixed as close as possible to its base. A row of interrupted mattress sutures, placed as close as possible to the clamp, joins the posterior wall of the appendage to the myocardium of the adjacent ventricle, just below the circumflex coronary vessels. Occasionally it is necessary to tie small branches of the coronary artery crossing this row of sutures. This does not cause any apparent damage as there is abundant coronary anastomosis in the dog heart (Moore⁵).

Preparation of the Auricular Appendage. The appendage can be opened on the anterior or posterior surfaces, by resection of the tip, or by bisecting it and everting the two resulting flaps. In this series longitudinal openings were cut along the borders of the appendage (Fig. 1). This creates two flaps which are joined at their tips, and therefore a valvular action can be expected. The adjacent walls are separated with fine curved forceps which are used to draw a paraffin-soaked thread through the two lateral incisions. This will

⁵ Moore, R. A., *Am. Heart J.*, 1930, 5, 743.

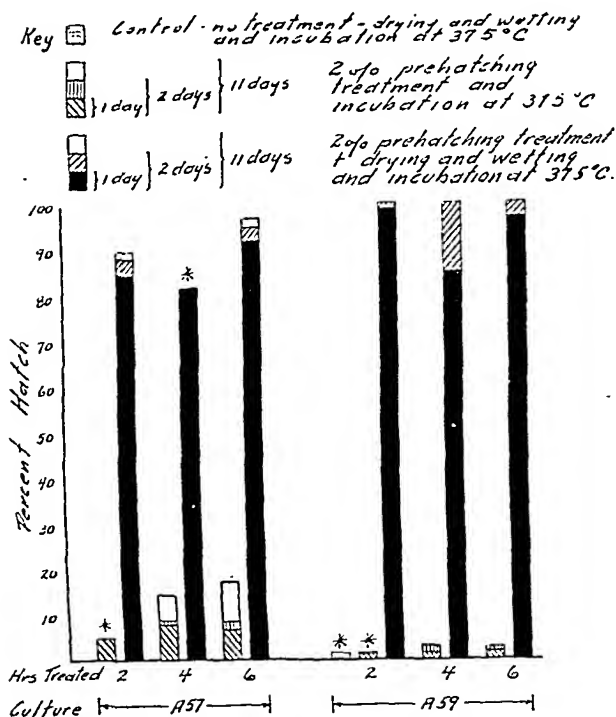


FIG. 1.

Bar graphs showing hatching results following treatment of *Ascaris* eggs in 2% "Clorox", 2% sodium hydroxide solution and subsequent drying, wetting and incubation.

* Per cent hatch did not increase after 1st day.

Under these conditions all eggs became embryonated in 24 days in most flasks. Cultures selected for hatching trials were at least 30 days old to allow for molting (Alicata⁵), had no eggs in stages earlier than the embryo, and showed at least 50% of the embryos motile.

Eggs to be used in hatching tests were treated at 37.5°C in a solution composed of 2% of the commercial sodium hypochlorite (5.25%) preparation "Clorox" and 2% sodium hydroxide. They were then washed five times in physiological saline. Eggs from individual worms were handled separately.

In the hatching tests 3 different methods were used:

1. Treated and washed eggs, in a few drops of physiological saline in shallow watch glasses, were incubated in moist chambers.

2. Suspensions of treated and washed eggs in shallow watch glasses were rapidly evaporated at 37.5°C with the aid of a fan. When salt crystals appeared throughout the suspension in each watch glass, the residue was rewet with saline and incubated in a moist chamber at 37.5°C.

3. Treated and washed eggs were centrifuged in physiological saline and the liberated larvae were available immediately after centrifuging.

Results. Some trials by method 1 resulted in all eggs hatching in 4 weeks of incubation at 40°C. However, most early-hatched larvae did not survive the length of time required to produce a high hatch.

By method 2 the liberation of larvae was more rapid, resulting in 100% hatches in 2 days in some trials. (Fig. 1). Up to 83.5% of the liberated larvae were alive in the hatching media at the end of 24 hours.

⁵ Alicata, J. E., U.S.D.A., Tech. Bull. No. 489, 1935, 44.

of penicillin are put into the thoracic cavity and the chest closed in the usual manner. The post-operative course is uneventful.

Observation. No regurgitation through the anastomosis was ever observed. As long as the clamp at the base of the auricular appendage remains closed, no ventricular pulsations in the appendage and no hemorrhage are seen. After the clamp is opened, blood from the atrium streams through the anastomosis and pulsates in rhythm with the atrium. Bleeding may now occur around stitch openings accidentally torn in the thin appendage wall, but it can easily be controlled.

This operation has been performed on 13 dogs of various size. One death occurred during the operation. The reason for the relatively good survival incidence could be

found in the fact that the heart during the whole period of surgical intervention remains fully active and its muscle supplied with fresh oxygenated blood. No attempt is made to operate in a bloodless field; rather the main consideration is given to reducing to a matter of seconds that phase of the operation when the ventricle is opened. This is achieved by means of the technic described above. The blood loss varies between 50 and 150 cc. After all, this technic deals with quickly taking care of a wound which has been produced in the heart before our very eyes, and that problem has long been solved in surgery.

The authors are indebted to Professor C. H. Best, who has shown continuous interest and provided facilities for these experiments. They are also grateful to Dr. J. Markowitz for his helpful advice and encouragement.

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Experimental Hatching of the Eggs of *Ascaris suum*.*

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Yoshida and Toyoda¹ reported the *in vitro* hatching of the eggs of *Ascaris suum* by direct incubation of the eggs for 5 days in a variety of media. Fenwick² did not secure hatching in several of the media reported by Yoshida and Toyoda,¹ but he consistently produced hatching in dilutions of Milton solution, a commercial sodium hypochlorite preparation. Fenwick³ secured living larvae by treating infective eggs with a 1:15 dilution of Milton solution for 12 hours at 38°C, expressing the larvae between a slide and coverslip, and then washing the liberated larvae with an experimental saline. Pick⁴ reported the hatch-

ing of the eggs of *A. megaloccephala* in a Ringer-horse serum solution 34 days after the liquid had evaporated, but during which time the residue had been kept moist. Pick⁴ further observed spontaneous hatching among eggs 21 days after transferring them from a Ringer-horse serum solution to a glucose agar medium.

As a preliminary step in the study of the culture requirements of the larvae of *A. suum*, it was necessary to develop a method of hatching *Ascaris* eggs that would provide large numbers of living larvae in a short time.

Materials and methods. Fertile eggs were secured from the mature female *Ascaris* worms from the small intestines of hogs. The eggs from individual worms were incubated at 23° to 25°C in 40 ml of 2% formalin in separate 250 ml cotton-stoppered Ehrlenmeyer flasks.

⁴ Pick, F., *Bull. de la Société de Pathologie exotique*, 1948, 44, 208.

* The early phase of the work was accomplished through a research fellowship awarded by the Zoological Society of San Diego under a grant from the Ellen Browning Scripps Foundation.

¹ Yoshida, S., and Toyoda, K., *Livro Jubilar do Professor Lauro Travassos*, 1938, 569.

² Fenwick, D. W., *J. Helminth.*, 1939, 17, 69.

³ Fenwick, D. W., *J. Helminth.*, 1939, 17, 211.

during 10 minutes of centrifugation at 3000 R.P.M. with a starting temperature of 40°C.

2. Batches of eggs from different worms

showed different hatching characteristics depending on the treatment strength and time, and influenced by subsequent procedure.

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Effect of Insulin, Adrenal Cortical Hormones, Salt and dl-Alanine on Carbohydrate Metabolism in Scurvy.*

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Previous work^{1,2} has shown that scorbutic guinea pigs display a disturbance of carbohydrate metabolism resulting in lowered glycogen stores in liver and body. Murray and Morgan¹ demonstrated that when glucose was fed the scorbutic animal failed to absorb it as readily as the normal animal. The blood sugar after this procedure was also abnormally high. Sigal and King³ had previously demonstrated a lowered glucose tolerance in scorbutic animals.

Banerjee⁴ has demonstrated in scorbutic guinea pigs an increase in adrenaline production. He attributed the disturbance in carbohydrate metabolism to an imbalance between adrenaline and insulin since the insulin content of the pancreas appeared to be markedly decreased in the scorbutic animal.⁵

Involvement of the adrenal gland in the changes incident to ascorbic acid deficiency has been suggested. Marked hypertrophy of this gland has been shown to accompany this deficiency. Sayers, Sayers, Liang and Long⁶ found that the injection of adrenotropic

hormone lowered both the cholesterol and ascorbic acid content of the adrenals of rats and guinea pigs suggesting that these 2 compounds are involved in the synthesis of cortical hormones. This hormone also greatly increased the deposition of liver glycogen in both species.

Intestinal absorption has been shown to be impaired in adrenalectomized rats^{7,8} and the improvement of this condition demonstrated by adding sodium chloride to the drinking water. There was a possibility that the decrease in absorption in the scorbutic animal could be improved by adding this compound to the drinking water.

MacKay and co-workers^{9,10} have shown that dl-alanine when fed to fasting mice or rats results in the formation of a considerable amount of hepatic glycogen. The possibility existed therefore, that the scorbutic animal could convert compounds of 3 C atoms to glycogen normally, and this might be some index of the adrenal cortical function.

This study was undertaken to determine the effect of insulin, adrenal cortical extract and additions of sodium chloride in the

* Published as Scientific Paper No. 786, College of Agriculture and Agricultural Experiment Stations, Institute of Agricultural Sciences, State College of Washington, Pullman, Wash.

¹ Murray, H. C., and Morgan, A. F., *J. Biol. Chem.*, 1946, **163**, 401.

² Hamme, B., *Acta Paediat.*, 1941, **28** Suppl.

³ Sigal, A., and King, C. G., *J. Biol. Chem.*, 1936, **116**, 489.

⁴ Banerjee, S., *J. Biol. Chem.*, 1945, **159**, 327.

⁵ Banerjee, S., and Ghosh, N. C., *J. Biol. Chem.*, 1947, **168**, 207.

⁶ Sayers, G., Sayers, M. A., Liang, T. Y., and Long, C. N. H., *Endocrinology*, 1946, **38**, 1.

⁷ Clark, W. G., and MacKay, E. M., *Am. J. Physiol.*, 1942, **137**, 104.

⁸ Anderson, E., Joseph, M., and Herring, V. V., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 477.

⁹ MacKay, E. M., Wick, A. N., and Carne, H. O., *J. Biol. Chem.*, 1940, **132**, 613.

¹⁰ MacKay, E. M., Wick, A. N., and Barnum, C. P., *J. Biol. Chem.*, 1941, **137**, 183.

TABLE I.

Results of Hatching Eggs of *A. suum* by 24 Hour Treatments in 2% "Clorox"-2% Sodium Hydroxide Solutions and Subsequent Centrifugation at 3000 R.P.M. for 10 Minutes. Starting Centrif. Temp. 40°C.

Orig. No.	Culture age (days)	% hatch
A76	37	77.1
A77	37	74.7
A78	37	65.8
A64	57	98.6
A61	57	99.1
A61	60	87.8
A76	67	74.2
A78	67	77.4
A77	104	70.5
A79	104	58.1

Method 3 produced hatching as high as 99.1%, and up to 87% of the liberated larvae were motile 24 hours after hatching. A treatment time of 24 hours in a 2% "Clorox"-2% sodium hydroxide solution followed by centrifugation at 3000 R.P.M.† for 10 minutes, gave consistently high hatches. (Table I). At the end of the 10-minute centrifuge period the temperature in the hatching tubes had dropped from 40°C to approximately 25°C.

Discussion. In a "Clorox"-sodium hydroxide solution the shells of *Ascaris* eggs are dissolved, so that after 24 hours many embryos are surrounded by thin elastic shell membranes only. At this stage motile embryos may produce prominent bulges in these elastic envelopes and may be liberated by their own activities. Later, a complete dissolving of the shells including the membranes may release the embryos in the treatment solution. The shells of eggs from the same worm do not dissolve at a uniform rate, and cultures of eggs from different worms show differences in the average speed at which the shells dissolve.

The hatching of treated eggs incubated in saline, as in method 1, appeared to be due to the penetration of the shell membrane by the active larvae and to some extent the result of osmotic pressure.

Hatching by drying and wetting treated eggs probably resulted from physical stresses,

set up in the chitinous shells when the eggs were dried, which caused the shells to fracture when rewet, rupturing the shell membranes beneath and liberating the larvae.

The pressure of membrane covered eggs against each other and the pressure of larvae against the sides of their membranous envelopes is a probable explanation of the hatching of treated eggs during centrifugation. Significant increases in hatching that appeared when the starting centrifuge temperatures were increased from 20° to 40°C were probably due to the liberation of larvae by their own efforts, due to greatly stimulated activity of the embryos at the higher temperature.

The motility of the larvae after hatching varied with the treatment time and with hatching conditions.

A study of photomicrographs in the report of Yoshida and Toyoda¹ revealed that the eggs pictured had no typical mammillated layer, but were essentially the same in appearance as those treated in "Clorox"-sodium hydroxide solution. The eggs were photographed in a 0.05% peptone solution, which in the present work was found to have no effect on the shells. These workers caution that all things used in hatching must be disinfected, and, although this phase of their technic is not described, it is possible that they used a sodium hypochlorite disinfectant, or some other chemical which had the same effect on the egg shells. If *Ascaris* eggs were permitted to remain in contact with such a disinfectant, the shells would be dissolved as shown in the photomicrographs and would probably have hatched in a variety of media as reported.

Summary. 1. Infective *Ascaris* eggs treated in "Clorox"-sodium hydroxide solution, hatched and produced viable larvae when they were subsequently:

(a) incubated in physiological saline at 37.5° and 40°C. Hatching occurred up to 100% in 4 weeks.

(b) dried, rewet and incubated in saline at 37.5°C. Hatching occurred up to 100% in 2 days.

(c) centrifuged at speeds from 2000 to 3000 R.P.M. Hatching occurred up to 99.1%

† 1958 gravities.

TABLE I.
Effect of Insulin, Adrenal Cortical Extract, Adrenaline, Sodium Chloride and dl-Alanine on Carbohydrate Metabolism in Scorbatic and Normal Guinea Pigs.

Treatment	No. of animals	Status	Glucose in intestinal tract, mg	Blood sugar mg, %	Liver glycogen %	Body glycogen %
None	9*	Normal	58 ± 20†	92 ± 6†	4.07 ± 0.20†	.528 ± 0.14†
	9	Deficient	166 ± 41	162 ± 20	2.66 ± 0.33	.439 ± 0.19
			t = 2.34‡	t = 3.33§	t = 3.71§	t = 3.90§
Insulin 20 units	8	Normal	17 ± 6	54 ± 7	1.70 ± 0.15	.353 ± 0.03
	8	Deficient	157 ± 53	143 ± 48	0.52 ± 0.19	.307 ± 0.08
			t = 2.64‡	t = 1.85	t = 4.10§	t = 0.52
Adrenal cortical extr., 3.5 ml	6	Normal	8 ± 4	135 ± 9	3.18 ± 0.38	.372 ± 0.03
	6	Deficient	132 ± 60	230 ± 59	1.85 ± 0.34	.324 ± 0.02
			t = 2.07	t = 1.61	t = 2.60‡	t = 1.33
Adrenaline 0.15 ml 1-1000	6	Normal		185 ± 9	0.92 ± 0.11	.183 ± 0.02
	6	Deficient		260 ± 30	0.62 ± 0.11	.133 ± 0.02
				t = 2.40	t = 2.0	t = 1.80
Sodium chloride 0.5% in drinking water	8	Deficient given NaCl	138 ± 42	225 ± 49	1.34 ± 0.18	.321 ± 0.03
	4	Deficient no NaCl	174 ± 50	293 ± 106	1.50 ± 0.29	.285 ± 0.02
			t = 0.55	t = 0.57	t = 0.47	t = 1.0
dl Alanine 1.4 g	8	Normal			0.79 ± 0.17	.309 ± 0.02
	8	Deficient			0.05 ± 0.02	.242 ± 0.03
					t = 4.0§	t = 1.60

* Figures taken from Table I, Murray, H. C., and Morgan, A. F., *J. Biol. Chem.*, 1946, 163, 401.

† Standard error.

‡ Indicates significance at the 5% level.

§ Indicates significance at the 1% level.

Snedecor, G. W., *Statistical Methods*, Collegiate Press, Inc., Ames, Iowa, 1938.

fasting levels about 7-fold after feeding glucose and 3-fold after lactate.

Respiration trials run after feeding 2.5 g of glucose to 5 animals fasted 24 hours and injected with 4 units of insulin resulted in an average figure of 0.42 cc of oxygen per square centimeter of body surface per hour. When this same procedure was carried out on the same animals depleted of their ascorbic acid supply until scurvy appeared a figure of 0.43 cc was secured. The lower glycogen stores in the deficient animals given insulin can not then be due to increased oxidation.

Injections of adrenal cortical extract failed to improve absorption or promote glycogen storage. Kendall¹⁴ noted that scorbatic animals given this extract survived longer than those not so treated but this did not cure the scurvy as the pathological lesions at death were the same whether animals received

the treatment or not. Ratsimamanga¹⁵ reported that scurvy produced an adrenal cortical deficiency and that injections of this hormone prolonged the survival period 1 to 3 weeks.

The addition of sodium chloride to the drinking water failed to improve any aspect of carbohydrate metabolism investigated in this study.

Adrenaline injections produced high blood sugar in both members of the pair; the level in the deficient animal was higher than that in the normal one. Lower glycogen levels were also found in the deficient animal, but the differences between the two groups were of no significance. Cori and Cori¹⁶ observed that when rats were given adrenaline in the post absorptive state high blood sugar resulted, carcass glycogen was lowered and some increase in liver glycogen occurred.

The deficient animal was apparently not as competent in converting dl-alanine to liver

¹⁴ Kendall, E. C., Mayo Clinic, Rochester, Minn., private communication.

¹⁵ Ratsimamanga, A. R., *Compt. rendu. Soc. de Biol.*, 1944, 138, 19.

¹⁶ Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 1925, 78, lxii.

drinking water on glucose absorption, blood sugar levels, glycogen content of liver and body in scorbutic guinea pigs. The effect of adrenaline on blood sugar, body and liver glycogen and the conversion of dl-alanine to glycogen were also investigated. The diverse roles of the adrenal and pancreatic hormones in ascorbic acid deficiency might thus be indicated.

Experimental. Young guinea pigs (550-600 g) of both sexes were used. They were paired as to sex and weight. Animals were pair-fed, the normal animal of each pair receiving the amount of basal diet that the deficient member had consumed the previous day. The basal diet used was a commercial rabbit food.[†] In addition to the diet all animals received a carotene in oil supplement sufficient to supply 1 mg of carotene daily. The normal animals were given a solution containing 10 mg of ascorbic acid 3 times weekly. All the animals were kept at a temperature of 75-80°F.

The animals kept on this diet without ascorbic acid supplements exhibited a decreased appetite in 19-24 days. At this stage scurvy was present as evidenced by extensive hemorrhages of the fascia of the musculature particularly of the legs. A rapid loss of weight accompanied the loss of appetite. A few days after inanition became apparent in the deficient member of the pair, the carbohydrate technic of Cori and Cori¹¹ was applied to both animals. They were allowed to fast for 24 hours and were then given a solution containing 2.5 g of glucose orally. One hour later each member of the pair was given an injection of 20 units of insulin intraperitoneally and sacrificed 6 hours later by the use of sodium amytal.

Animals used for the experiments with adrenal cortical extract were treated similarly to those in the insulin experiment with the exception that the deficient animal only received an intraperitoneal injection of $\frac{1}{2}$ ml of adrenal cortical hormone[‡] at hourly inter-

vals 7 times in accordance with the assay procedure of Reinecke and Kendall.¹²

Animals used for the investigation of the effect of sodium chloride were treated as were the deficient animals in other groups except that a 0.5% solution of this compound was given for drinking water during the depletion period.

The effect of adrenaline was studied on animals prepared as were those in the above groups. After a 24 hour fast both the normal and deficient member of the group received an intraperitoneal injection of 0.15 ml of 1 to 1000 saline solution of adrenaline. They were sacrificed 3 hours later; blood sugar, liver and carcass glycogen determined.

In the investigation involving dl-alanine the deficient and normal animals were treated as were those in other groups. After a 24 hour fast a solution containing 1.4 g of dl-alanine was fed orally. Animals were sacrificed 12 hours later; liver and body glycogen determined.

The analytical procedure and methods as outlined by Murray and Morgan¹ were followed in all experiments.

Results and Discussion. The results of the analytical work are shown in Table I.

The injection of insulin failed to facilitate the storage of glycogen in the deficient animal; intestinal absorption was still impaired and blood sugar levels were high. Both the normal and deficient animals given insulin tended to show lower liver glycogen stores than animals not so treated; carcass glycogen did not seem to be affected. Cori and Cori¹¹ using the same technic with normal rats also found substantially lower amounts of liver glycogen in animals given insulin but body glycogen was comparable to that in the controls. Stetten and Klein¹³ applying this same treatment to rats likewise found low liver glycogen levels, but large increases in carcass glycogen over

‡ Whole extract of the adrenal cortex—75 g/ml donated by Dr. E. C. Kendall, Mayo Clinic, Rochester, Minn.

¹² Reinecke, R. M., and Kendall, E. C., *Endocrinology*, 1942, **31**, 573.

¹³ Stetten, D., Jr., and Klein, B., *J. Biol. Chem.*, 1945, **159**, 593.

[†] Purina rabbit pellets.

¹¹ Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 1926, **70**, 557.

identified by their herd number, and the trials by Roman figures.

Cow 798 was 7 years old, weighed 400 kg, and was in the 5th month of lactation when, for the trial III, she was injected intravenously with 50 ml of a phosphate solution isotonic with blood and containing 15 millicuries of P³². The same amount of a similar solution, but containing 30 millicuries of P³², was injected into cow 890 which was 3 years old, weighed 370 kg, and had calved 5½ months before the trial started. Cow 798 produced daily 9.5 liters of milk; cow 890 8.7 liters. These production rates are means for the second to the seventh day after injection. The radioactive solution was injected into the jugular vein through plastic tubes otherwise used to insulate wires and known to electricians as "spaghetti". This method, which will be described in detail later, causes a minimum of excitement, and provides therefore a good chance that no "hot" solution is spilled or leaks into the tissues around the vein. The cows were milked just before injection, at 1, 2, 6 and 12 hours after injection, and later twice a day. The radioactivity of the milk was measured in 0.1 ml samples pipetted on 1" diameter disks of lens cleaning paper, dried on 1¼" diameter aluminum disks and then fixed with a spray of an alcoholic shellac dispersion.

Results. The concentration of P³² in milk reached a maximum during the period of 3 to 8 hours after injection. This maximum amounted to 1.21% of the injected dose per

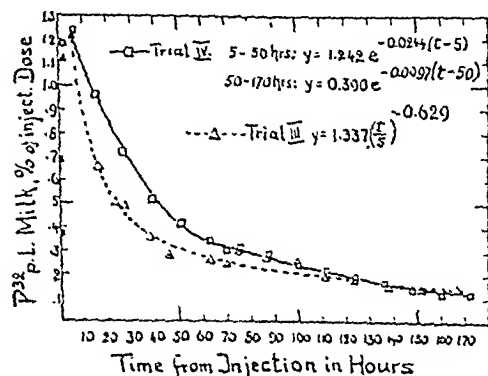


FIG. 1.
Radioactivity in milk.

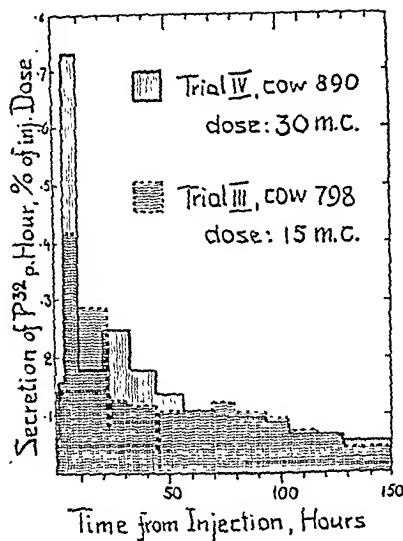


FIG. 2.
P³² secretion.

liter milk of cow 798 in Trial III and to 1.24% per liter milk of cow 890 in Trial IV.

Fig. 1 shows the P³² concentration in milk as a function of time from injection.

The relative rates of secretion of P³² in milk are illustrated by Fig. 2. The histogram gives the results of the direct measurements in each period.

The average daily secretion of P³² in milk as percentage of the injected dose for the first to the 7th day after injection amounted to 7.4, 4.9, 2.8, 2.1, 1.6 and 1.4% respectively.

In a week, cow 798 (Trial III) secreted, in 66 liters of milk, 20% of the injected P³²; cow 890 secreted, in 58 liters of milk, 23% of the injected dose.

Discussion. Cows injected intravenously with 15 and 30 millicuries of P³² respectively produced "hot" milk almost immediately. The radioactivity of the milk of both cows reached the same peak at about 5 hours after injection when 1 liter of milk contained 1.2% of the injected P³². Doubling the injected dose of P³² thus changed neither the time at which the maximum activity in milk was reached nor the relative height of the maximum P³² concentration in milk as percentage of the injected dose.

At the time of maximum activity in the milk, the inorganic P (trichloroacetic acid fil-

glycogen as was the normal.

Summary. The injection of insulin or adrenal cortical extract in guinea pigs deficient in ascorbic acid did not materially alter the disturbance in carbohydrate metabolism characteristic of scorbutic animals; glycogen levels were still low, intestinal absorption impaired and blood sugar levels high after feeding glucose. Oxygen consumption of animals fed glucose then injected with insulin before and after depletion was un-

changed. The addition of sodium chloride to the drinking water of deficient animals failed to improve any aspect of carbohydrate metabolism studied. Adrenaline given to both members of the pair produced higher blood sugar levels in the normal than in deficient animals and slightly higher levels of liver glycogen. Normal animals demonstrated much greater ability to convert dl-alanine to liver glycogen than did the deficient.

16717

Secretion in Cow's Milk of Intravenously Injected Radioactive Phosphorus P³²*

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Since phosphorus plays a major role in intermediary metabolism, especially in biological energy transformations, the measurement of phosphorus transfer and turnover

rates in domestic animals is important in research on the basic factors of food utilization.

*This paper is the first report on a cooperative project, "Metabolic Research with Isotopes," started two years ago on the Davis campus among members of the Divisions of Chemistry and Animal Husbandry, and now including workers of other divisions. The authors take this opportunity to thank the colleagues who were instrumental for a promising start of this tracer work. Dr. H. Reiber of the Division of Chemistry provided us with the first sample of P³². He also helped us with the analytical work during our first trial, as did Dr. J. K. Loosli, on sabbatical leave from Cornell University. Drs. M. E. Gardner and Charles G. Patten of the Division of Mathematics and Physics made many measurements of radioactivity for us and trained us in the use of the Geiger counter borrowed from Dr. W. B. Hewitt, Division of Plant Pathology. The generous help and advice of members of the Department of Medical Physics at Berkeley, especially Dr. H. Jones, has been most encouraging. Last but not least, we are glad to acknowledge the faithful and ever-ready help of Mr. Th. Chernikoff, technician in the Division of Animal Husbandry.

[†] At present at the Department of Medical Physics, Berkeley.

This report deals with the rate at which intravenously injected P³² is secreted in the milk, and its concentration in milk, as a function of time. These data are of particular interest for planning tracer experiments with large animals and for appraising cows as producers of labeled compounds, such as casein, for other trials. Comparing the secretion rate and concentration in milk of P³² with that of Sr⁹⁰, mentioned earlier by Erf and Pecher,¹ leads to the tentative conclusion that the phosphorus stored in the body is more mobile than the calcium.

The excretion of P³² in feces and urine, and specific activities in blood and other body constituents will be discussed in later papers in connection with the results of additional work still under way.

Method. Two lactating Jersey cows were used for the two trials on phosphate secretion reported in this paper. The same cows were, and still are, used in other trials with P³². To simplify later comparisons, the cows will be

¹ Erf, L. A., and Pecher, Ch., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 762.

16718 P

Androgenic Secretion by Tumors of the Mouse Adrenal Cortex.

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Tumors of the human adrenal cortex may be responsible for either androgenic or estrogenic activity, although it is more common for such neoplasms to give evidence of androgenic secretion.¹ Adenomas of the adrenal cortex of mice may be induced in certain stocks by gonadectomy,^{2,3} and in one stock, the NH, they occur spontaneously, primarily in females.^{4†} In contrast to the human, the mouse neoplasms have been found to secrete primarily estrogen in most instances.¹⁻³ It was consequently of great interest to the authors to find that in the Bagg albino stock the majority of tumors induced in mice of both sexes secreted primarily androgenic hormone, although it is likely that both estrogenic and androgenic substances are produced simultaneously.

All of 10 males castrated at weaning age possessed tumors of the adrenal cortex 2 years following operation. In 5 of the 10, the seminal vesicles were very large and filled with secretion; in 4 others the seminal vesicles

were developed beyond the castrate type. Submaxillary glands⁵ and renal corpuscles⁶ were histologically male in most instances.

Ten months following ovariectomy 3 of 4 Bagg albino females had cortical tumors with which there was an associated androgenic activity. The vaginal epithelium was only 3-4 cell layers thick and the superficial lining cells were high columnar, the cytoplasm being filled with mucous. This picture is indicative of combined estrogenic and androgenic secretion.⁷ Seminal vesicle grafts⁸ of weanling Bagg albino males had been made into both axillae of 2 of the females 57 days before autopsy. All 4 grafts were greatly hypertrophied, and filled with secretion. The renal corpuscles and submaxillary glands of these females were of the male type. The fourth female of this group had cortical tumors of histologic structure similar to that found in the other 3 ovariectomized mice. In this case, however, the reproductive tract showed signs of estrogenic stimulation, and the renal corpuscles and submaxillary glands had not become masculinized.

Summary. A majority of gonadectomized male and female mice of the Bagg albino stock developed adrenal cortical tumors which secreted androgenic hormone. There is evidence that both androgenic and estrogenic hormone may be produced simultaneously by these tumors.

* This investigation has been aided by grants from the National Cancer Institute, The Jane Coffin Childs Memorial Fund for Medical Research, and the Cancer Fund of the Graduate School of the University of Minnesota.

¹ Cahill, G. F., In *Endocrinology of Neoplastic Diseases*, 1947, Oxford University Press, New York.

² Gardner, W. U., *Cancer Research*, 1941, **1**, 632.

³ Wooley, G., Fekete, E., and Little, C. C., *Endocrinology*, 1941, **28**, 341.

⁴ Kirschbaum, A., Frantz, M. F., and Williams, W. L., *Cancer Research*, 1946, **6**, 707.

[†] The authors have also observed spontaneous tumors of the adrenal cortex in old, intact Bagg albino mice of both sexes and in aged females of the CBA strain.

⁵ Lacassagne, A., *C. R. Soc. de Biol.*, 1940, **133**, 180.

⁶ Pfeiffer, C. A., Emmel, V. M., and Gardner, W. U., *Yale J. Biol. and Med.*, 1940, **12**, 493.

⁷ Korenechevsky, V., and Hall, K., *J. Path. and Bact.*, 1937, **46**, 681.

⁸ Hill, R. T., *Endocrinology*, 1937, **21**, 633.

trate) was relatively more active than the organic P. This conclusion is drawn from the observation that even 10 hours after injection, the organic P³² in milk amounted to less than 20% of the total P³², whereas during the 5th to 7th week of lactation, according to Van Slyke and Bosworth² 20% of the milk P is present in organic form, practically all in casein. Aten and Hevesy³ have reported such a lag in the specific activity of casein P as compared to inorganic P in goat milk. However, 5 hours after injection, the specific activities were already equalized. Goats may have higher turnover rates than cows. Experiments are under way to get more definite information on the partition of P³² among the various constituents of the milk. Despite the lag mentioned, cows are good producers of labeled casein. From milk collected for 3 days after injecting 40 millicuries of P³² per cow, we have prepared casein with a radioactivity of 2 microcuries per gram.

The peak of P³² activity in the milk of our cows was reached at nearly the same time after intravenous injection as the peak of Sr⁹⁸ in milk of two Holstein cows in the earlier trials of Erf and Pecher (l.c.). Sr⁹⁸, however, reached a higher maximum relative concentration, namely 2.2% of the injected dose per liter milk, and then decreased faster than the P³² in our trials. For 4½ days after injection of the Sr⁹⁸, the two cows secreted 8% and 11% respectively of the injected isotope in the milk, whereas in 4 days after injection of P³² 15.1 and 17.9% respectively were secreted by our 2 cows.

The different results with Sr (representing Ca) and P might be expected if cows had a greater storage capacity for P than for Ca

in terms of the corresponding amounts secreted in milk. Such an explanation, however, would be unsatisfactory, in view of the relative concentrations of these elements in milk and body. According to Van Slyke and Bosworth (l.c.), milk contains 0.103% P and 0.143% Ca. Using figures of Hogan and Nierman⁴ for steers, one may estimate that the body of cattle contains 0.72% P and 1.36% Ca. If this estimate is applicable to our cows, then the ratio (P in body : P in milk) is only ¾ of the ratio (Ca in body : Ca in milk).

The different behavior of Sr (substituting for Ca) and P in lactating cows may be explained by the assumption that the P stored in the body is on the average more mobile than the Ca stored. A faster storage of P would explain the lower peak in milk, and a faster exchange of the stored P would account for the subsequent relatively higher level of P³² in the milk.

Summary. Intravenous injection of 15 and 30 millicuries of P³² respectively into 2 lactating cows produced a maximum relative activity per liter of milk of 1.2% of the injected dose at about 5 hours after injection. The relative activity per liter milk is plotted against time after injection and regression equations are presented for interpolating the data. A week after injection, the milk still contains slightly over 0.1% of the injected P³² per liter.

Casein with a radioactivity of 2 microcuries per gram was prepared out of milk collected during 3 days after injection of 40 millicuries of P³².

A histogram shows the relative rate of P³² secretion in milk. In a week, the 2 cows secreted in their milk 20 and 23% respectively of the injected P³².

² Van Slyke, D. D., and Bosworth, A. W., *J. Biol. Chem.*, 1915, **20**, 141.

³ Aten, A. H. W., Jr., and Hevesy, G., *Nature*, 1938, **142**, 111.

⁴ Hogan, A. G., and Nierman, J. L., *Univ. Mo. Res. Bull.*, 1927, **107**, 45.

TABLE I.
Sex distribution in *Rana sylvatica*.

Treatment	♀	♀	♂	♂	♂
Testosterone propionate					
1000 $\mu\text{g/l}$	—	—	—	—	30
200 "	—	—	—	—	38
40 "	—	—	—	—	46
10 "	1	—	—	2	131
2 "	—	—	—	—	77
1 "	13	2	3	7	66
0.1 "	45	2	4	6	40
0.01 "	47	—	2	2	37
Controls	68	—	4	—	44

47 : 69 in favor of either sex, as calculated by 3 P.E. or 2 S.E. The observed distribution of 48 males and intersexes : 68 females falls within these limits but is close to the extremes and, together with the occurrence of 4 hermaphrodites, may therefore indicate a slight deviation toward the semi-differentiated racial type.

Discussion. It is logical to assume that the tadpole does not utilize all of the hormone available in its ambient environment. This would certainly appear to be indicated judging from Foote's⁶ measurements of the slow rate of disappearance of estrone from the aquarium water in which *Rana pipiens* tadpoles were kept. Although it may well be that the animals are able to concentrate the absorbed hormone to critical levels, we observe at any rate that they exhibit a remarkable ability to "detect" exceedingly minute amounts of androgen to which they respond with a marked alteration in morphogenesis. This testifies to the extreme lability of the embryonic sex inductor system in anurans.

The method outlined might serve as an assay procedure for ascertaining the presence of very small quantities of testosterone not otherwise determinable. A series of experimental groups treated with successively diluted aliquots of the preparation in question would indicate that proportion of the total which contained the minimal effective dose for male differentiation.

Table II summarizes data on sensitivity of some of the more widely studied vertebrates to testosterone propionate. Because of the

different modes of hormone administration, the differing extents of changes produced as well as total weight of the test animal, and, presumably, differences in economy in utilizing the hormone, we must of course regard such comparisons with some degree of reservation. The tests cited indicate more recently reported lowest levels at which an effect can still be obtained; credit is not here necessarily given to the originator of the test. Literature on quantitative bioassays has by now become impressive in volume, and includes some disagreement on threshold dosages. For purposes of comparison, the threshold dose for masculinization of *Rana sylvatica* is expressed as $\mu\text{g/ml}$ since this amount of solvent is considerably closer to the volume administered to other test animals than is the "per liter" designation.

In examining these data, it is apparent that the anuran larva displays a much greater reactivity to testosterone propionate as compared with other vertebrates studied, and that the nature of the response, involving the primary sex characters, is a relatively more drastic one.

There are indications of the existence of species differences in response among anurans. Treatment of a small number of *Hyla arenicolor* larvae with the 2 $\mu\text{g/liter}$ dose resulted in approximately equal numbers of males and females, plus a few hermaphrodites.

Summary. An amount of testosterone propionate as small as 1 $\mu\text{g/liter}$ of aquarium water, a concentration of 1 : 1,000,000,000, is sufficient to induce male development in a

⁶ Foote, C. L., *J. Exp. Zool.*, 1941, 86, 291.

Testosterone Propionate Minimum for Induction of Male Development in Anurans; Comparative Data from Other Vertebrates.*

BEATRICE MINTZ. (Introduced by Emil Witschi.)

From Whitman Laboratory of Experimental Zoology, The University of Chicago.

After the discovery^{1,2} that administration of male sex hormone to anuran tadpoles can induce testicular development in genetic females, it became of some interest to study the sensitivity of the system in terms of the smallest quantity of androgen which could effect such a change. In previous investigations in which tadpoles were raised from the time of hatching to metamorphosis in water containing graded concentrations of testosterone propionate, male differentiation irrespective of genetic constitution was observed in *Rana sphenoccephala*, *Rana pipiens*, and *Pseudacris triseriata* at 0.01 mg (10 μ g) of hormone per liter of aquarium water, a concentration of 1:100,000,000.^{3,4} From all indications, however, it appeared that the minimal effective dose might well be considerably below any so far employed. Such has since been found to be the case (preliminary report by Mintz and Witschi⁵).

Materials and Methods. *Rana sylvatica* eggs were collected near New Haven, Conn. in March and April and treatments were initiated 6-8 days after hatching. Controls preserved at this time were entirely undifferen-

tiated as to sex. Groups of 50-100 larvae, including untreated controls, were each kept in 10 liters of aerated water. Solutions of crystalline testosterone propionate† in 95% ethyl alcohol were added daily from calibrated syringes to the following concentrations in different groups: 1,000, 200, 40, 10, 2, 1, 0.1, and 0.01 μ g/liter. The alcohol volume measured 0.15 ml/liter of water. These animals were preserved in Bouin's fixative approximately 30 days later during metamorphosis when one foreleg perforated the opercular covering. Individuals that died were preserved only if they appeared to be still in good condition. The temperature of the amphibian room averaged 20°C but occasionally dropped down to 16°C.

Results. As seen in Table I, tadpoles exposed to 2 μ g or more of hormone/liter emerge exclusively as phenotypic males indistinguishable from control males, with the exception of 2 male-like intergrades and one female at 10 μ g/liter. It is possible that the latter non-conformist was accidentally transferred from one of the lower dosage jars. The amount of 2 μ g/liter, still inducing complete masculinization of all genetic females, corresponds to a concentration of 1:500,000,000. One half of this quantity, 1 μ g/liter or 1:1,000,000,000, represents the threshold value for masculinization inasmuch as it produces a preponderance of males and masculine intersexes.

Among the controls, we observe a moderate excess of females. In a population of this size, the probability of obtaining a deviation from the expected 1:1 ratio of males : females (actually 58 males : 58 females) due to the error inherent in random sampling would bring the acceptable range to the extremes of

† The author wishes to thank the Ciba Pharmaceutical Products, Inc., for supplying testosterone propionate (Perandren).

* The experimental part of this work was conducted at the State University of Iowa and supported by grants from the National Research Council, Committee for Research in Problems of Sex, under the administration of Dr. Emil Witschi. The author wishes to thank Dr. L. V. Domm for providing accommodations for completing the histological portions of the study at Whitman Laboratory.

1 Gallien, L., *C. R. Acad. Sci.*, 1937, **205**, 375.

2 Witschi, E., and Crown, E. N., *Anat. Rec. Suppl.*, 1937, **70**, 121.

3 Witschi, E., *Cold Spring Harb. Sympos. on Quant. Biol.*, 1942, **10**, 145.

4 Witschi, E., Foote, C., and Mintz, B., *Anat. Rec. Suppl.*, 1942, **84**, 455.

5 Mintz, B., and Witschi, E., *Anat. Rec. Suppl.*, 1946, **96**, 30.

large majority of *Rana sylvatica* larvae raised in such a solution from hatching to metamorphosis. The relationship of these results to similar work on other vertebrates is discussed.

The author wishes to acknowledge the assistance of Miss Jane Elchlepp of the Department of Zoology, The State University of Iowa, in the maintenance and treatment of some of the experimental groups.

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Concerning the Relation Between Pituitary Adrenocorticotrophin and the Circulating Blood Platelets.

MONTE A. GREER AND BRUCE R. BROWN. (Introduced by R. O. Greep.)

From the Joseph H. Pratt Diagnostic Hospital and the Department of Medicine, Tufts Medical School, Boston, Mass.

Although it has been known for some time that infection, trauma, hemorrhage, asphyxia, etc. result in rather marked alterations in the number of circulating blood platelets, the exact mechanism controlling the number of platelets is as yet unknown. With the discovery by Dougherty and White¹ that pituitary adrenocorticotrophin caused a dissolution of lymphoid tissue and a peripheral lymphopenia a new line of investigation was opened up. Since the conditions under which changes in the platelets have been reported are those which would stimulate a release of pituitary ACTH, and since this hormone has been shown to affect other blood elements through the adrenal, it did not seem too unreasonable to believe that the platelets might also be at least to some extent under the control of corticotrophin.

Consequently experiments were undertaken in both rats and humans to determine whether any effect of ACTH upon the blood platelets could be demonstrated. As far as could be determined by these experiments, there is no effect upon the platelets of either species by this hormone.

Experimental. Sixteen 100-150 g, hooded, male rats were injected subcutaneously with 80 mg of a suspension of acetone-dried hog pituitary after two control counts had been made, and determinations of the platelets*

and RBC were made from the capillary tail blood every hour for 8 hours. No significant change was seen to occur in either blood element.

Six rats were injected in a similar manner with 40 mg of hog pituitary powder every morning for 3 days. Again there was no significant change in the number of platelets.

Four rats were injected with 10 mg of a purified pituitary corticotrophin preparation and counts made every hour for 8 hours with similarly negative results.

Following these experiments a preparation of pituitary ACTH† which was quite pure was made available to us, and it was decided

* Three methods were used to count the platelets: (1) that of Rees and Ecker²; (2) a direct chamber count using 3.8% sodium citrate as the diluting fluid; (3) an indirect count of a wet preparation containing platelets stained with brilliant cresyl blue. The direct counts were used chiefly on the rats and the indirect on the human subjects.

² Rees, H. M., and Ecker, E. E., *J.A.M.A.*, 1923, 80, 621.

† Kindly supplied by Dr. John R. Mote of the Armour Laboratories, Chicago, Ill. It was believed that each vial in the lot supplied (Lot G-59703-II) contained the equivalent of 12 mg of the Armour ACTH standard. After the completion of these experiments Dr. Mote informed us that on re-assay each vial contained only the equivalent of 5.2 mg of their standard. The doses given in this paper conform to the final assay.

¹ Dougherty, T. F., and White, A., *Endocrinology*, 1944, 35, 1.

TESTOSTERONE THRESHOLD FOR MALE DEVELOPMENT

TABLE II.
Testosterone Propionate, Minimal* Effective Dosages.

Test animal	Diagnostic effect	Method of administration	No. of days	Dosage	Reference
Brown Leghorn capon	Comb growth	Inj. (intramus.) daily	4-5	5-25 $\mu\text{g}/\text{d}$	Koch ⁷
Brown Leghorn capon	" "	Local application, daily	10	.01-.1 $\mu\text{g}/\text{d}$	"
English sparrow capon	Bill blackening	Inj. (intramus.) daily	6	.2 $\mu\text{g}/\text{d}$	Witschi ⁸
English sparrow capon	" "	Local application, daily	4-16	.06 $\mu\text{g}/\text{d}$	Pfeiffer, Hooker, and Kirschbaum ⁹
Rat σ (8 days old)	Prostate	Inj. daily	6	1 $\mu\text{g}/\text{d}$	Priebe ¹⁰
Rat σ (8 days old)	Prostate, seminal vesicles	" "	6	1 $\mu\text{g}/\text{d}$	"
Rat σ , pregnant	Masculinization of secondary sex char's. of σ embryos	Inj. once	—	2500 μg	Greene and Ivy ¹¹
Hamster σ , pregnant	Masculinization of secondary sex char's. of σ embryos	" "	—	1000 μg	Bruner and Witschi ¹²
Mouse σ , pregnant	Masculinization of secondary sex char's. of σ embryos	" "	—	1000 μg	Turner ¹³
Zona aglytica	Male development	Immersed	30†	0.001 $\mu\text{g}/\text{ml}$	

* Not all workers on mammals listed have tried doses below those given, so that the minima in some cases may be lower.

† Minimum for testosterone rather than testosterone propionate. Positive results with 2 $\mu\text{g}/\text{d}$ of testosterone propionate for 4-5 days but minimum not determined.

‡ The critical time has not been determined. Possibly the results could be read sooner.

⁷ Koch, F. C., 1946, personal communication.⁸ Witschi, E., *Anat. Rec. Suppl.*, 1943, **87**, 478.⁹ Pfeiffer, C. A., Hooker, C. W., and Kirschbaum, A., *Endocr.*, 1944, **31**, 389.¹⁰ Priebe, D., *Physiol. Zool.*, 1944, **17**, 377.¹¹ Greene, R. R., and Ivy, A. C., *Science*, 1937, **80**, 200.¹² Bruner, J. A., and Witschi, E., *Am. J. Anat.*, 1946, **70**, 293.¹³ Turner, C. D., *J. Morphol.*, 1939, **65**, 353.

Effect of Diisopropyl Fluorophosphate (DFP) on the QO_2 of the Rabbit Cerebral Cortex.

SYDNEY ELLIS.* (Introduced by G. S. Eadie.)

From the Biochemistry Section, Medical Division, Army Chemical Center, Md.

Several reports indicate that diisopropyl fluorophosphate (DFP), or related compounds, may be of clinical value in view of their prolonged inhibition of cholinesterase activity. When injected into humans, stimulation of cholinergically innervated tissues has been noted. The central nervous system also appears to be affected.^{1,2} Since 'central' effects have not been noted when neostigmine is administered by the usual routes,³ or in large doses by continuous infusion,⁴ the suggestion has been made that this difference in the action of the two agents is due to the greater fat solubility of DFP in contrast to neostigmine.^{1,2}

Another possibility exists, i.e., that DFP, in addition to inhibiting cholinesterase, might affect the metabolism of the brain. This has been investigated in rabbits injected with DFP in doses sufficient to inactivate cholinesterase.

Methods. Normal rabbits were sacrificed as controls by injecting air intravenously. Diisopropyl fluorophosphate at a concentration of 1:1000 freshly dissolved in physiological saline was injected intravenously in amounts stated in the table. The animals were used immediately following death or, if not dead 30 minutes after the injection of

DFP, they were sacrificed by injecting air intravenously. Immediately after death the cranium was opened and the cerebrum removed intact.

Cerebral cortex slices were removed from one cerebral hemisphere by the tissue slice technic of Deutsch.⁵ The remaining hemisphere was homogenized in 0.025 M sodium bicarbonate solution⁶ with the Potter all-glass homogenizer and made up to a final dilution of 1:10 with the bicarbonate solution.

Cholinesterase activity was determined by Ammon's method⁷ with 0.5 cc of the 1:10 brain homogenate in a final concentration of 0.015M acetylcholine bromide in 0.025M sodium bicarbonate at 38°C. The gas phase was 5% CO_2 and 95% N_2 .

The oxygen uptake, expressed as cu mm oxygen utilized per mg of dry weight of tissue per hour (QO_2), was determined on cortical slices suspended in 3 cc of a salt mixture of the following composition⁸: 124.6 mM sodium chloride; 4.01 mM potassium chloride; 1.04 mM calcium chloride; 0.52 mM magnesium chloride; 10 mM phosphate buffer, pH 7.4; 0.20 g% anhydrous glucose. The dry weights of the tissues were obtained after the tissues were heated at 105°C for at least 16 hours.

The data of Table I demonstrate that DFP does not change the oxygen uptake of cerebral cortex slices of rabbits treated with this compound, as compared with untreated rabbits, even when acetylcholine hydrolysis by the cerebrum is completely inhibited. This does not eliminate the possibility of a more subtle

* Formerly Captain, Sanitary Corps. A.U.S. Present address: Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, N. C.

¹ Comroe, J. H., Jr., Todd, J., Gammon, G. D., Leopold, I. H., Koelle, G. B., Bodansky, O., and Gilman, A., *Am. J. Med. Sci.*, 1946, **212**, 641.

² Grob, D., Harvey, A. M., Langworthy, O. R., and Lillenthal, J. L., Jr., *Bull. Johns Hopkins Hosp.*, 1947, **81**, 257.

³ Harvey, A. M., Lillenthal, J. L., Jr., Grob, D., Jones, B. F., and Talbot, S. A., *ibid.*, 1947, **81**, 267.

⁴ Acheson, G. H., and Ellis, S., unpublished data.

⁵ Deutsch, W., *J. Physiol.*, 1936, **87**, 56.

⁶ Ellis, S., and Root, M. A., *Fed. Proc.*, 1944, **3**, 70.

⁷ Ammon, R., *Arch. ges. Physiol.*, 1930, **231**, 456.

⁸ Jandorf, B. J., and Williams, R. H., *Am. J. Physiol.*, 1944, **141**, 91.

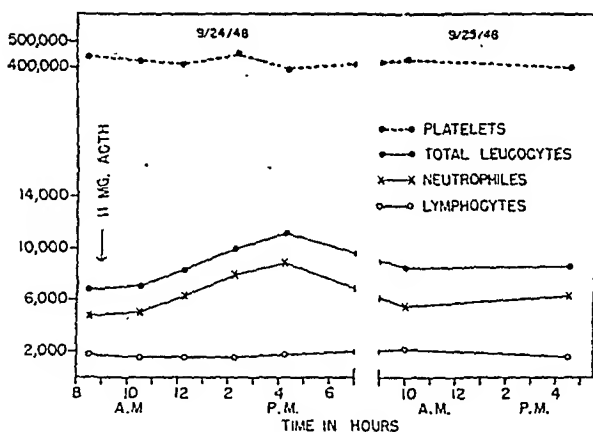


Fig. 1.

The effect of a single, intramuscular injection of 11 mg of ACTH

to test this in human subjects. Following a control determination of all blood elements, 11 mg of this preparation were injected intramuscularly into a normal male subject every 4 hours for 24 hours. A white count, differential, and platelet counts were performed every 2 hours for the first day and in the morning and afternoon of the second day.

The same procedure was followed in a young woman with idiopathic thrombocytopenic purpura who had not had a favorable response to splenectomy.† (It was reasoned that an increase in the platelets might show up much more readily in a patient in whom they were quite low originally.)

Two normal young women were given 11 mg of ACTH intramuscularly and the above counting procedure followed.

One young woman with hypopituitarism, which had presumably resulted from postpartum pituitary necrosis 3 years previously, was given 11 mg of ACTH in the morning and 7 mg in the afternoon and counts made as above.

In no instance could any significant change in the number of circulating platelets be observed, although there was a definite and usually quite striking rise in the total white count and the polymorphonuclear leukocytes.

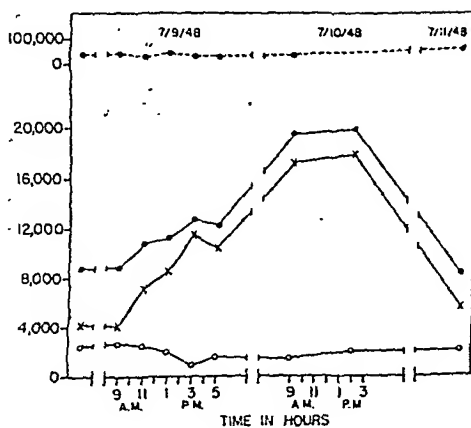


Fig. 2.

The effect of 66 mg of ACTH on a patient with idiopathic thrombocytopenic purpura. 11 mg were given intramuscularly every 4 hours for 24 hours beginning at 8 A.M. July 9. The legend corresponds to Fig. 1.

It is interesting that the number of circulating lymphocytes remained relatively constant, no true lymphopenia being observed in any instance. In the patient with hypopituitarism the increase in the polymorphonuclear leukocytes was much less marked than in the other subjects.

Summary. Under the above experimental conditions, no effect of pituitary adrenocorticotrophin upon the number of circulating blood platelets could be detected.

† Patient of Dr. William Dameshek.

appeared to have inhibitory effects.

This fraction was prepared by extracting finely minced sheep cardiac muscle with acetone in the cold, evaporating the solvent, lyophilizing and dissolving the dry residue in 10 volumes of Tyrode's solution. From 660 g of fresh cardiac muscle the yield was approximately 10 g of residue. The following is a report on preliminary experiments dealing with the effects of this material.[†]

Effect on Normal Cells. Third passage hanging-drop cultures of chick heart fibroblasts were divided and one half of each culture was used as the test object, the other half as the control. Each was planted in a separate D-3.5 Carrel flask containing 0.5 cc of chicken plasma, 1 cc Tyrode's solution and one drop of chick embryonic extract. After 48 hours 0.5 cc of the test solution was added as a supernatant layer to the test culture and 0.5 cc of Tyrode's solution to the control. The pH of both control and test cultures was adjusted to from 6.5 to 7.0. The effect of the test solutions as compared to the controls was observed for from 2 to 4 days.

The residue of the acetone extract of the sheep heart, redissolved in Tyrode's solution, markedly inhibited the growth of chick fibroblasts as no increase whatsoever in surface area was observed after this material had been added in dilutions up to 1:500. The cells, however, were not killed, for when the cultures were washed with Tyrode's solution and transplanted into fresh medium they began to grow and to maintain a rate of growth eventually equaling that of the controls.

Effect on Tumor Cells. To test the effects of this extract on tumor cells, cultures of a mouse breast tumor were also studied. This neoplasm arose spontaneously in a C3H mouse 2 years ago, and while originally exhibiting all the features of a mammary adenocarcinoma, it has gradually become sarcomatous in character.[‡] The tumor was cultivated in Carrel flasks in the same type of coagulum used for the normal cells, but 0.3 cc of rabbit

serum and 0.4 cc of mouse embryo extract were added as a supernatant fluid phase. The latter was changed every 4 days and the cultures transplanted to other Carrel flasks every 10 days.

Second passage cultures were divided into equal parts and the halves were planted in a separate Carrel flask to be used either as test or control objects. All cultures were maintained in medium 4 or 5 days, during which time satisfactory growth of the tissue was observed. On the fourth or fifth day, the supernatant fluids were removed from all the cultures and replaced in the experimental series with the residue from the acetone solution that had been redissolved in Tyrode's solution to give a final dilution of 1:500. In the sister halves, or controls, 0.5 cc of Tyrode's solution was added. A complete cessation of growth was obtained in the tumor cultures treated with extract, whereas the sister cultures continued to show good growth.

Effect on Bacteria. The effectiveness of certain animal tissues in producing bacteriostasis and in converting *Staphylococcus aureus* R into an avirulent form has been reported by Nutini and coworkers in several publications;³⁻⁶ and in view of these findings it is of interest to examine the effect of our acetone extract on certain bacteria. One gram of the crude residue was dissolved in 10 cc of Tyrode's solution and applied in serial dilution to standard cultures of organisms. It inhibited the growth of tubercle bacilli in the Tween albumin medium of Dubos and Davis⁷ in further dilution of 1/40 and was effective against the same organism cultivated in oleic acid medium when the 10% solution of the crude material was further diluted 1/160.

Complete inhibition of growth of H *Streptococcus* C 203 occurred when the 10% Ty-

³ Nutini, L. G., and Kreke, C. W., *J. Bact.*, 1942, **44**, 661.

⁴ Nutini, L. G., Kreke, C. W., and Seroeder, Gr. M. P., *J. Bact.*, 1943, **50**, 177.

⁵ Nutini, L. G., and Lynch, E. M., *J. Exp. Med.*, 1946, **84**, 247.

⁶ Nutini, L. G., and Lynch, E. M., *J. Bact.*, 1946, **52**, 681.

⁷ Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

[†] The crude material was prepared by The Armour Laboratories.

[‡] H. Toolan, personal communication.

TABLE I.
Oxygen Uptake and Cholinesterase Activity of
Brain of Normal and DFP-Treated Rabbits.

DFP mg/kg i.v.	Cholinesterase activity cu mm CO/30 min/ 0.5 cc ² homogenate	QO ₂
0.0	233	11.2
0.0	299	10.9
0.0	246	9.6
0.0	222	10.2
0.0	244	9.9
0.0	233	10.3
0.0	239	10.5
0.0	221	9.9
Avg	242	10.3
0.5	4	9.9
0.5	4	10.2
0.5	4	10.6
1.0	4	8.8
1.0	-0.7	10.4
1.0	-1.1	11.4
Avg	2	10.2

All values are averages of duplicate or triplicate determinations.

effect on brain metabolism. It does show, however, that DFP has no appreciable effect on the normal oxygen uptake of cerebral cortical slices, and that cholinesterase activity is not essential for such oxygen uptake.

Discussion. Some related studies of the effect of DFP on enzymes other than esterases have been reported. Webb⁹ found that the

following enzyme systems were not affected by DFP at concentrations of M/500 to M/2000: cytochrome oxidase, succinic dehydrogenase, hexokinase, Robison ester glycolysis by muscle powder, choline, dehydrogenase, catalase, carboxylase, pyruvate, peroxidase, lactic dehydrogenase of muscle, diaphorase, liver alcohol dehydrogenase, and carbonic anhydrase. Hunt¹⁰ has stated that the normal oxygen uptake by liver homogenate and the additional oxygen uptake due to added ethyl butyrate are not inhibited noticeably by DFP.

The increased respiration of rat brain slices observed by Levy¹¹ when the tissue was in concentrations of eserine salicylate between 1:500 and 1:1000 are attributable to the enormous concentration of the drug. It may be that the salicylate ion is being metabolized at a sufficient rate at this high concentration to produce the increased oxygen uptake attributed to eserine.

Summary. When injected into rabbits in lethal doses, diisopropyl fluorophosphate had no appreciable effect on the oxygen uptake of cerebral cortex slices of these animals even though cholinesterase was completely inhibited.

¹⁰ Hunt, C. C., personal communication.

¹¹ Levy, J., *Bull. soc. chem. biol.*, 1946, **28**, 338.

⁹ Webb, E. C., *Biochem. J.*, 1948, **42**, 96.

16722 P

In vitro Studies of a Growth Inhibitory Fraction Obtained from Adult Sheep Cardiac Muscle.*

R. S. HOFFMAN, JAMES A. DINGWALL AND WILLIAM DEW. ANDRUS.
(with the technical assistance of Irene Jacob.)

From the Department of Surgery, New York Hospital and Cornell University Medical College,
New York City.

In earlier papers^{1,2} we have reported on the

* This work was supported by a grant furnished by the Committee on Growth of the National Research Council.

¹ Hoffman, R. S., and Dingwall, J. A., *Surg., Gynec. and Obst.*, 1944, **79**, 103.

² Hoffman, R. S., Dingwall, J. A., and Andrus, W. DeW., *Ann. Surg.*, 1946, **124**, 1125.

growth stimulating effects of a Tyrode's solution extract of animal tissue as demonstrated both *in vitro* and when applied topically to wounds in dog and man. In the course of attempts to fractionate adult animal tissue extracts in order to determine the factors responsible for this stimulation, one crude fraction has been of particular interest because it

were modifications of those described by Reinecke, Ball, and Samuels.⁶ The rats were brought to a full feeding of 26 cc per rat per day on the 5th day. After the rats had been force-fed for 2 weeks the adrenal glands were removed by the procedure of Ingle and Griffith.⁷ In the control animals the adrenal glands were exposed but were not damaged. Asepsis was successfully maintained in these operations. All of the animals were given a solution of 1% sodium chloride to drink during all phases of the experiments. The animals were housed in an air-conditioned room in which the temperature was maintained at 74 to 78°F and the humidity at 30 to 35% of saturation.

Twenty-four-hour samples of urine were collected at the same hour each day and were preserved with thymol with added citric acid (1 g per sample) to insure the acidity of the urines for nitrogen analysis. The determination of urinary nonprotein nitrogen was by the micro-Kjeldahl procedure as follows: proteins were precipitated as the salts of tungstic acid by the Folin-Wu procedure. The

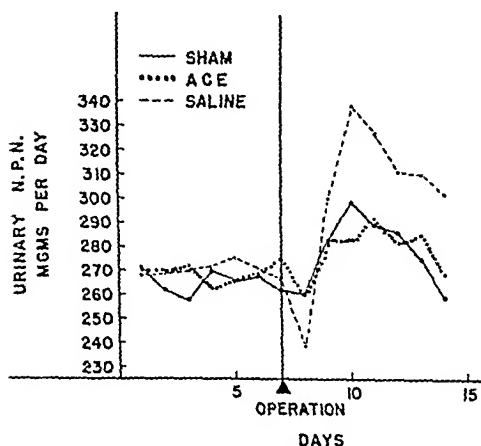


FIG. 1.

The effect of adrenal cortex extract upon the level of urinary nitrogen immediately following adrenalectomy. Averages of 8 rats per group.

⁶ Reinecke, R. M., Ball, H. A., and Samuels, L. T., *Proc. Soc. Exp. Biol. and Med.*, 1939, 41, 41.

⁷ Ingle, D. J., and Griffith, J. Q., Chapter 16, *The Rat in Laboratory Investigation*, J. B. Lippincott Co., Philadelphia, 1942.

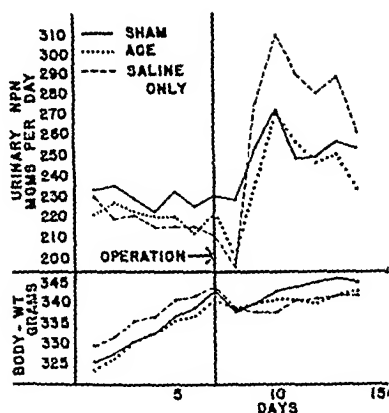


FIG. 2.

The effect of adrenal cortex extract upon the level of urinary nitrogen immediately following adrenalectomy. Averages of 10 rats per group.

organic matter was oxidized by sulfuric acid and hydrogen peroxide. Copper sulphate and sodium sulphate were used as catalysts. The ammonia was distilled off into a standard acid solution and titrated with standard base.

Experiments and results. In Experiment 1 (Fig. 1) 24 rats were observed during a control period of 2 weeks. At this time 8 rats were adrenalectomized and were given 4 cc of beef adrenal extract (Upjohn) per day for 7 days; 8 adrenalectomized rats received injections of 4 cc of 0.9% sodium chloride solution for 7 days; and 8 sham-operated rats received injections of 4 cc of 0.9% sodium chloride solution for 7 days. The adrenalectomized rats showed some decrease in urinary nitrogen during the first 24 hours with a subsequent rise. The increase in nitrogen loss was significantly greater in the saline-injected adrenalectomized animals than in either the treated adrenalectomized group or the sham-operated controls.

Experiment 2 (Fig. 2) was identical with Experiment 1 except that 30 rats were used and it was carried out during the summer months instead of early winter. The relative changes following operation were identical with the results of Experiment 1. The level of urinary nitrogen was significantly lower than for Experiment 1 during all phases of the experiments.

Discussion. These data support our earlier

rode's solution was diluted 1/16, Staphylococcus was inhibited in dilution of 1/32 (crude material 1/320) and *E. coli* at 1/16.

Other investigators⁸⁻¹¹ have reported the extraction of substances inhibiting cell growth from liver and other sources by methods dif-

ferent from those reported here, and it is not yet clear whether the substance which we have used corresponds in its physical and chemical properties to any of those previously described.

Summary. Acetone extract of sheep cardiac muscle lyophilized and redissolved in Tyrode's solution inhibits the growth of chicken fibroblasts and mouse breast tumor cells *in vitro*. A similar inhibitory activity of varying degree is exhibited against standard strains of tubercle bacilli, hemolytic *Streptococcus aureus* and *E. coli*.

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Effect of Adrenal Cortex Extract Upon the Urinary Nitrogen of Rats Following Adrenalectomy.

DWIGHT J. INGLE AND MILDRED C. PRESTRUD.

From the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

The adrenal cortex has been characterized as a regulator of protein metabolism. It is generally true that the adrenally insufficient animal excretes smaller than normal amounts of urinary nitrogen and that the administration of adrenal cortex extract or of the 11-oxy steroids favors an increased loss of nitrogen¹. The present report concerns an exception to the above rule. Others are known.²⁻⁵

In studies on the force-fed rat it was noted^{4,5} that following adrenalectomy the animals which were given saline to drink without additional treatment excreted more urinary nitrogen than did similar animals which were treated with adrenal cortex extract or than did sham-operated rats. The

present study confirms and extends these preliminary observations.

Methods. Male rats of the Sprague-Dawley strain were maintained on stock diets of Purina Dog Chow (Experiment 1) and Archer Dog Pellets (Experiment 2). When the rats reached a weight of approximately 300 g they were adapted to the force-feeding of a medium carbohydrate diet by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The technic of force-feeding and the diet (Table I) used

TABLE I.
Medium Carbohydrate Diet.

Constituent	G
Cellu flour (Chicago Dietetic Supply)	120
Osborne & Mendel salt mixture	40
Dried yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Vitamin K (2 methyl-1,4-naphtho-quinone)	100 mg
Mazola oil	200
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
Water to make total of	2000 cc

¹ Long, C. N. H., Katzin, B., and Fry, E. G., *Endocrinology*, 1940, **26**, 309.

² Koelsche, G. A., and Kendall, E. C., *Am. J. Physiol.*, 1935, **113**, 335.

³ Berman, D., Sylvester, M., Hay, E. C., and Selye, H., *Endocrinology*, 1947, **41**, 253.

⁴ Ingle, D. J., and Oberle, E. A., *Am. J. Physiol.*, 1946, **147**, 222.

⁵ Ingle, D. J., Ward, E. O., and Kuizenga, M. H., *Am. J. Physiol.*, 1947, **149**, 510.

were modifications of those described by Reinecke, Ball, and Samuels.⁶ The rats were brought to a full feeding of 26 cc per rat per day on the 5th day. After the rats had been force-fed for 2 weeks the adrenal glands were removed by the procedure of Ingle and Griffith.⁷ In the control animals the adrenal glands were exposed but were not damaged. Asepsis was successfully maintained in these operations. All of the animals were given a solution of 1% sodium chloride to drink during all phases of the experiments. The animals were housed in an air-conditioned room in which the temperature was maintained at 74 to 78°F and the humidity at 30 to 35% of saturation.

Twenty-four-hour samples of urine were collected at the same hour each day and were preserved with thymol with added citric acid (1 g per sample) to insure the acidity of the urines for nitrogen analysis. The determination of urinary nonprotein nitrogen was by the micro-Kjeldahl procedure as follows: proteins were precipitated as the salts of tungstic acid by the Folin-Wu procedure. The

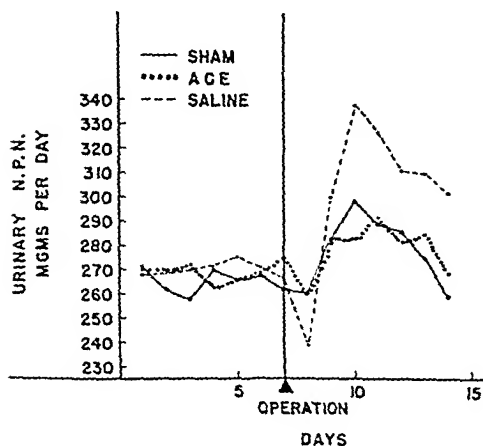


FIG. 1.

The effect of adrenal cortex extract upon the level of urinary nitrogen immediately following adrenalectomy. Averages of 8 rats per group.

⁶ Reinecke, R. M., Ball, H. A., and Samuels, L. T., *Proc. Soc. Exp. Biol. and Med.*, 1939, 41, 44.

⁷ Ingle, D. J., and Griffith, J. Q., Chapter 16, *The Rat in Laboratory Investigation*, J. B. Lippincott Co., Philadelphia, 1942.

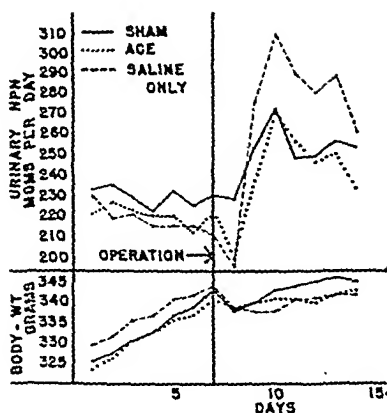


FIG. 2.

The effect of adrenal cortex extract upon the level of urinary nitrogen immediately following adrenalectomy. Averages of 10 rats per group.

organic matter was oxidized by sulfuric acid and hydrogen peroxide. Copper sulphate and sodium sulphate were used as catalysts. The ammonia was distilled off into a standard acid solution and titrated with standard base.

Experiments and results. In Experiment 1 (Fig. 1) 24 rats were observed during a control period of 2 weeks. At this time 8 rats were adrenalectomized and were given 4 cc of beef adrenal extract (Upjohn) per day for 7 days; 8 adrenalectomized rats received injections of 4 cc of 0.9% sodium chloride solution for 7 days; and 8 sham-operated rats received injections of 4 cc of 0.9% sodium chloride solution for 7 days. The adrenalectomized rats showed some decrease in urinary nitrogen during the first 24 hours with a subsequent rise. The increase in nitrogen loss was significantly greater in the saline-injected adrenalectomized animals than in either the treated adrenalectomized group or the sham-operated controls.

Experiment 2 (Fig. 2) was identical with Experiment 1 except that 30 rats were used and it was carried out during the summer months instead of early winter. The relative changes following operation were identical with the results of Experiment 1. The level of urinary nitrogen was significantly lower than for Experiment 1 during all phases of the experiments.

Discussion. These data support our earlier

observations^{4,5} that the administration of adrenal cortex extract to force-fed rats immediately following adrenalectomy tends to suppress the post-operative increase in urinary nitrogen as compared with that of the untreated animal. The adrenalectomized rat usually shows a temporary decrease in urinary nitrogen which is not shown by sham-operated animals. This may possibly reflect a difference in the breakdown of thymus and other lymphoid tissue or it may reflect an increase in nitrogen retention in the body fluids. Noble and Toby⁸ reported a temporary decrease in loss of nitrogen after adrenalectomy followed by a rise above the pre-operative values.

Koelsche and Kendall² found that adrenal cortex extract tended to inhibit the catabolic effect of thyroxin in the adrenally insufficient dog. Berman *et al.*³ noted that regeneration of liver is suppressed in the adrenalectomized rat and that regeneration is supported by treatment with adrenal cortex extract.

The possibility must be considered that adrenal cortex extracts favor anabolism under certain conditions because of the presence of androgenic steroids. However, such extracts

have never been shown to possess androgenic properties.

The average level of urinary nitrogen was higher in all phases of Experiment 1 than in Experiment. 2 Such shifts in the average level of constituents of blood, urine and tissues are not uncommon in this laboratory. The reasons have never been elucidated beyond the demonstration that even small variations in temperature are important. Values on the nitrogen content of our fluid diets, on standards and unknowns have remained uniform. Because unknown variables influence the results of experiments which are thought to be under rigid control, it is essential that all control studies be done in parallel with the experimental study.

Summary. Two experiments were carried out as follows. Force-fed rats given saline to drink were subjected to adrenalectomy or to sham operations. Half of the adrenalectomized rats were treated with 4 cc of beef adrenal extract per day. All of the animals showed an increase in urinary nitrogen following operation but it was significantly less in those animals which either had their adrenal glands intact or were treated with adrenal cortex extract.

16724

Augmentation of Femoral Venous Flow in the Dog by Electrical Stimulation of Muscles.

V. L. TICHY AND B. W. SHAW. (Introduced by F. R. Mautz.)

From the Department of Surgery, Western Reserve University, Cleveland, Ohio.

The procedure was performed 6 times on 2 mongrel dogs, weighing approximately 11 kg. They were anesthetized with intravenous nembutal. Eighteen mg of heparin was given intravenously at the beginning of the procedure. The femoral vein was exposed and cannulated. On one dog a straight glass cannula was used. On the other a T-shaped cannula was inserted, so that no backing up of blood was allowed between periods of flow. The diameter of the T-cannula was smaller

than that of the straight one; consequently the minute flow was less. The blood was caught in a sterile graduated cylinder, filtered and reinfused at the end of the experiment.

The stimulation was achieved by means of faradic current. The intensity of the current and the placing of the electrodes on the limb were so arranged that a gentle contraction of the muscles of the whole limb was obtained. The stimulation was intermittent, 2 seconds of exercise being alternated with 3 seconds of

rest. The total period of repeated stimulation was 30 seconds and the flow per minute was calculated.

The flow of blood from an open free flowing cannula was caught and measured, both during a period of control when the limb was resting and flaccid, and during a period of stimulation as described above. Near the end of one of the procedures, the cannula became partially occluded by clotted blood, and the flow was reduced to a slow drip. The flow during both exercise and rest was also recorded under these circumstances. Several observations were recorded on each dog, and averages were computed as shown in the tables. Measurements during stimulation were alternated with measurements during rest in an effort to eliminate after-effects of stimulation on blood vessels.

Results. Measurements are recorded in the

¹ Krogh, August, *The Anatomy and Physiology of the Capillaries*, New Haven, Yale University Press, 1927.

² Büllbring, Edith, and Burn, J. H., *J. Physiol.*, 1939, 95, 203.

TABLE I.

No obstruction	At rest cc	With stimulation cc
Dog No. 1	45	90
	39	79
	33	74
Avg	39	81
Dog No. 2	30	53
	27	50
Avg	28.5	51.5

table. The venous return was increased from 80 to 100% by stimulation of the muscles when the cannula was flowing freely. When there was a partial obstruction, the increase was considerably greater, in one observation.

Conclusions. From these observations it is concluded that the venous flow of blood from a limb is increased by muscular contractions elicited by periodic electrical stimulations. The increase in flow was from 80 to 100%. Results suggest that increased blood flow is one of the reasons why similar methods, when applied to a large number of postoperative patients, have effected a marked reduction in the incidence of venous thrombosis.

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Protection of Gastric Mucosa of the Rat against Ulceration by Prefeeding with Protein Hydrolysates.

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The beneficial effects which Co Tui and his associates¹ reported to have resulted from the addition of large amounts of protein hydrolysates to the dietary of patients with gastric or duodenal ulcers have been amply corroborated by subsequent clinical experience. However, no adequate explanation of the action of such preparations has as yet been found. It has been thought that the buffering action² of the protein hydrolysates and/or the re-

sultant improvement in the nitrogen balance^{3,4} may be responsible for the beneficial effects observed. Opinions based on clinical studies have been expressed in favor of each of the above mechanisms, but the evidence presented cannot be regarded as conclusive. While the administration of protein hydroly-

² Levy, J. S., and Siler, K. A., *Am. J. Digest. Dis.*, 1942, 9, 354.

³ Hodges, H. H., *Gastroenterology*, 1947, 8, 476.

⁴ Kenamore, B., Lonergan, W., and Shy, J. C., *Gastroenterology*, 1948, 10, 177.

¹ Co Tui, Wright, A. M., Mulholland, J. H., Galvin, T., Bareham, I., and Gerst, G. R., *Gastroenterology*, 1945, 5, 5.

sate to ulcer patients has given encouraging clinical results, the effect of the hydrolysate on peptic ulcer and the mechanism of its action, as far as we know, have not been investigated experimentally.

We have described a method for the uniform production of ulceration in the rumen of the rat.⁵ With this method ulceration develops as a result of the action of acid gastric juice accumulating in the empty stomach after pyloric ligation. In addition, a certain minimal concentration of pepsin and of acid is necessary for the development of ulceration, since neutralization of the acid or inactivation of the pepsin prevents ulceration.⁵ We realize that rumen ulcers in the rat represent lesions of the wall of a cavity lined with squamous epithelium which in their pathogenesis may differ somewhat from ulcers of the glandular mucosa. However, as we⁵ have pointed out previously, this method is valuable in studying the resistance of the gastric mucosa to acid-pepsin ulceration under various experimental conditions. This method has been employed by others⁶⁻⁹ as well as by ourselves¹⁰ for assaying antacid and anti-ulcer agents. By its means we have studied the effect of protein hydrolysates on the development of experimental ulcers in the rat. In this communication we report results which indicate that the fortifying of an otherwise adequate diet with protein hydrolysate affords definite protection to the gastric mucosa of the rat against the ulcerative action of acid gastric juice.

Experimental method and results. In the present study 106 rats were used. All the

animals were females of the Wistar strain, bred in our own colony. After weaning, they were maintained on the Rockland Diet.* We confined our investigation to one sex in these experiments on account of the difference in the weight curves of the two sexes, and particularly since Zucker and Zucker¹¹ have shown that the more rapidly growing male requires more protein in the diet than the female. At the beginning of the experiments the weights of the animals ranged from 100 to 120 g. The females in each litter were divided into 3 groups, one control and 2 experimental. All the animals were fed the Rockland Diet *ad libitum* and the amount of food consumed by each animal during the experimental period of 28 days was recorded.

Two commercial preparations of protein hydrolysate were used as dietary supplements, each in a separate experimental series. Thus, in addition to the colony diet, animals in one series received by stomach tube 2 cc of hydrolysate A† in the morning and also in the late afternoon 6 days a week for a period of 4 weeks (28 days). The animals in the second experimental series received similar 2 cc quantities of a suspension of hydrolysate B‡ which has a nitrogen content equivalent to that of hydrolysate A. Since hydrolysate A contains 22% of sucrose, a similar solution of sucrose alone was administered as a supplement to the animals in the control series to substitute for the protein hydrolysate. The experiments were done on 10 animals at a time, namely,

* The "Rockland Rat Diet" contains 24.53% of protein, 4.67% of fat, and 49.32% of carbohydrate, according to the statement of the manufacturers (Aready Farms Milling Company).

† Zucker, T. F., and Zucker, L., *Proc. Soc. Exp. Biol. and Med.*, 1944, 55, 136.

‡ Hydrolysate A, supplied by Sharp and Dolme, Inc., contains an equivalent of 35% of protein, as calculated from the nitrogen content ($N \times 6.25$), and in addition 22% of sucrose. Control sucrose solution contained added vitamins present in hydrolysate A.

§ Hydrolysate B, supplied by Mead, Johnson and Co., contains an equivalent of 75% of protein, as calculated from the nitrogen content ($N \times 6.25$). Caloric value: 1 g = 3.75 C.

⁵ Shay, H., Komarov, S. A., Fels, S. S., Meranze, D., Gruenstein, M., and Siplet, H., *Gastroenterology*, 1945, 5, 43.

⁶ Wick, A. N., Irish, A. J., Pauls, F., and MacKay, E. M., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 40.

⁷ Pauls, F., Wick, A. N., and MacKay, E. M., *Gastroenterology*, 1947, 8, 774.

⁸ Risley, E. A., Raymond, W. B., and Barnes, R. H., *Am. J. Physiol.*, 1947, 150, 754.

⁹ Morris, C. R., Grossman, M. I., and Ivy, A. C., *Am. J. Physiol.*, 1947, 148, 382.

¹⁰ Shay, H., Komarov, S. A., Siplet, H., and Gruenstein, M., *Am. J. Digest. Dis.*, 1947, 14, 99.

on 5 which had received supplemental hydrolyzed protein and on 5 controls. All the animals treated similarly have been grouped together in one series and the different series will be referred to as hydrolysate A-treated, hydrolysate B-treated, and sucrose-treated controls respectively.

Some modifications of our previously described technic were adopted in this study. Following the suggestion of Pauls, Wick and Mackay,⁷ we used brief ether anesthesia during the operative period, not supplementing it with urethane as we had done in our original studies.⁵ This resulted in more rapid recovery of the animal, a much earlier restoration of active gastric secretion, and a more rapid production of ulcers (97% ulceration in 6 hours when 2 cc of saline were instilled into the stomach after pyloric ligation).

The animals in all 3 series underwent the same preliminary treatment, which was as follows. After 48 hours' starvation the animal was placed under light ether anesthesia and the pylorus was ligated. The stomach was lavaged with distilled water, warmed to 38°C, and 2 cc of saline were then introduced. Each animal was placed in a separate clean cage, and was sacrificed 6 hours later.

In this study we routinely inspected the full length of the esophagus in each animal. We believe this to be essential, for in some cases perforating ulcers of the esophagus develop rather early. This causes a large part of the gastric juice to be lost from the stomach, and as a result the residual volume of gastric juice within the stomach becomes so small that it is insufficient to bathe the rumen and cause ulceration. A few cases of perforating esophageal ulcers occurred in our experiments and these were discarded.

Table I shows the number of animals with rumen ulceration and the mean values for the composition of the gastric contents of the animals in each series. It is clear that the fortifying of the already adequate diet with protein hydrolysate produced a rather striking increase in the resistance of the rumen mucosa to the destructive action of the very potent gastric juice. In a considerable pro-

TABLE I.
Incidence of Rumen Ulcers and Composition of the Gastric Contents in Rats Pretreated with Protein Hydrolysates and in Sucrose-Treated Controls.

Series	Ulcer incidence		Animals used†	Composition of gastric contents (mean values)					Pepsin Conc. Act. (Mott units)
	No. of animals*	%		Volume (ml/hr per 100 g body wt)	pH	Acidity Total Free (m.eq/l)	Cl (m.eq/l)		
Sucrose control	35		32	.801	1.19	80	157	81	10.7
	34	97	31	.803	1.19	80	157	80	10.8
	1	3	1	.741	1.05	94	166	108	9.6
Amisate treated	46		44	.840	1.15	86	158	77	10.4
	28	59	26	.854	1.15	85	157	80	10.3
	18	41	18	.783	1.14	87	158	73	10.5
Protolysate treated	30		30	.868	1.14	88	156	61	7.1
	22	73	22	.898	1.14	86	156	65	7.3
	8	27	8	.793	1.11	94	156	49	6.5

* % of animals with ulcers in each series was calculated from all animals used.

† For analysis of gastric contents several animals were not available due to accidents or heavy contamination of the gastric contents with blood.

TABLE II.
Severity of Ulceration in Protein Hydrolysate-Treated* Rats and in Sucrose-Treated[†] Controls.

Series	No. of animals	Rumen lesions per animal				P†
		Single mean	Coalescent mean	Mean	Total S.D.†	
Sucrose-treated controls	34	6.2	8.5	14.7	11.60	0.003
Protein hydrolysate-treated*	50	4.2	4.6	8.8	8.30	

* Amisate and protolysate treated series combined.

† Standard deviation.

‡ Probability of both groups belonging to same population.

TABLE III.
Average Daily Food Consumption and Calorie Intake and Average Weight Gain per Animal During Experimental Period (28 Days).

Series	No. of animals	Food intake (g)				Wt (g)	
		Protein	Carbohydrate	Fat	Caloric intake	Initial	Gain
Sucrose-treated (control)	32	a	3.27	6.51	0.61	47	110
		b	—	0.68	—	—	38.8
		c	100%	100%	100%	—	—
Amisate-treated	44	a	2.90	5.79	0.55	47	111
		b	1.17	0.68	—	—	43.7
		c	125%	89.9%	90.1%	—	—
Protolysate-treated	30	a	2.89	5.72	0.54	44	111
		b	1.16	—	—	—	38.9
		c	124%	79.8%	88.4%	—	—

a: Intake with Rockland diet.

b: Intake with hydrolysate (values for protein equivalent calculated from nitrogen content ($N \times 6.25$)).

c: Total intake expressed as percentage of intake of sucrose-treated control series.

portion of the protein hydrolysate-treated animals, namely, 41% in the hydrolysate A-treated series and 27% in the hydrolysate B-treated series, no ulceration developed, while of the control animals only 3% had no ulcers. This difference was found to be highly significant statistically. Furthermore, those animals in the hydrolysate-treated series that did develop ulcers showed significantly fewer lesions per animal than the animals in the sucrose-treated series (Table II).

The adopted classification of the rumen lesions was, from the very nature of the ulcerative process, somewhat arbitrary. However, under magnification of 10.5 X, the differentiation between "single" and "coalescent" multiple lesions is not difficult. The former are characterized by even, circular margins; the latter by scalloping or irregularity in outline and, as a rule, by greater dimensions. The relatively greater reduction in the "coal-

escent" ulcers in comparison with single lesions in the hydrolysate treated animals increases the significance of the results.

The most striking feature of these experiments is the absence of any significant differences between the hydrolysate A- or the hydrolysate B-treated series and the sucrose-treated controls in respect of the volume, acidity, and peptic power of the gastric contents. The difference in the incidence of ulceration manifestly cannot be attributed to concurrently lower digestive power of the gastric juice in the hydrolysate-treated animals. We believe that our results indicate this effect of protein hydrolysate to represent a true increase in the mucosal resistance and that it is to be distinguished from the protective effect obtained with other agents, e.g., antacids, which act by depressing the digestive power of the gastric juice. The increased resistance of the mucosa in the hy-

drollysate-treated animals was not due to a greater caloric intake, since the average amounts of food consumed daily by the animals in all series were isocaloric, although the diets differed in composition. This is illustrated by the data presented in Table III, which also shows the proportions of protein, carbohydrate, and fat in the different diets, so that the food intake of the hydrolysate A- and the hydrolysate B-treated series may be compared with that of the sucrose-treated control series. The rate of growth was adequate in all groups and no statistically significant difference was found between the weight gain of the sucrose-treated controls and that of the protein hydrolysate-treated animals.[§] This indicates that the protein in the Rockland Diet was adequate for the animals at the weight levels at which the experiments were started, and that the addition of protein hydrolysate did not result in any significant increase in the nutritional value of this diet.

Nevertheless, these experiments show that the prefeeding of approximately 25% more hydrolyzed protein to the experimental animals than was fed to the controls for the relatively short period of 28 days resulted in a very marked increase in the resistance of the rumen mucosa to peptic ulceration. This may be regarded as a true prophylactic action. The only other preparation that has been reported to be effective in the prevention of peptic ulceration is enterogastrone, as demon-

strated in experimental studies on dogs.¹²

It is noteworthy that this prophylactic effect of protein hydrolysate was exerted against the ulcerating action of acid and pepsin, the agents which were shown to be responsible for the type of experimental ulcer produced by our method in the rat, and which also are generally accepted as factors of primary importance in the development of peptic ulceration in man. All concepts regarding the etiology of gastric and duodenal ulcers in man recognize that factors other than acid and pepsin are also involved. Among these are included differences in the resistance of tissues to the destructive action of gastric juice. If the prophylactic action of protein hydrolysate, which is demonstrated in this study for the rat, should be found to apply also to man, the first step in the solution of the all-important problem of ulcer prevention will have been accomplished by the simple expedient of fortifying the diet. Experiments are now being carried out to determine whether the addition of native proteins to the diet will produce the same effect as hydrolysates.

Summary. The supplementing of an already adequate diet with protein hydrolysates in amounts equivalent to 25% of additional protein produced in rats a marked increase in the resistance of the gastric rumen to peptic ulceration without concurrent changes in the volume, the acidity, or the peptic power of the gastric contents. This protective effect was not accompanied by any significant changes in the rate of growth of these animals as compared with animals on a control diet supplemented with sucrose alone.

[§] Hydrolysate A-treated *versus* Hydrolysate B-treated: $t = 1.85$, d. f. = 75, $P = 0.06$. Hydrolysate A-treated *versus* sucrose-treated control: $t = 1.64$, d. f. = 81, $P = 0.10$. Hydrolysate B-treated *versus* sucrose-treated control: $t = 0.02$, d. f. = 64, $P = >0.55$.

¹² Hands, A. P., Greengard, H., Preston, F. W., Fauley, G. B., and Ivy, A. C., *Endocrinology*, 1942, 30, 905.

Technical Procedures for the Study of Organogenesis *in vitro* in Amblystoma.

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The purpose of this report is to present the technical procedures which have been developed in a study of the organogenesis and differentiation of small pieces of embryonic amphibian tissue. Explants from larval *Amblystoma punctatum* (*Amblystoma maculatum* (Shaw)) were used. The methods employed were modifications of existing tissue culture technics.

Among the many studies of explanted tissues *in vitro*, relatively few have dealt with potentialities and capabilities of explants to differentiate into formed structures, such as organs or organ systems. Fischer¹ first reported endodermal epithelial aggregates which differentiated into cellular structures of the alimentary tract. These structures, called *intestinal organisms*, have been reported occasionally since. Using the Ekman technic of explantation to tap water, Stöhr² reported several cases where heart-like structures developed from Bombinator explants. Some of these beat rhythmically within their epithelial sacs. Examination of published photographs of these experiments reveals little in the way of histological differentiation, or of the formation of anatomically proper lumina. All were very small. (Technical details are lacking in the report of Stöhr.) True morphogenesis has been demonstrated by Fell³ in a series of remarkable papers dealing with the developmental mechanics of bones of the chick *in vitro*. Fell succeeded in effecting the development, in an essentially normal manner, of rudiments of femur, sternum and limb

joints from explants of 4 to 9 day avian embryos. Holtfreter⁴ has reported, in connection with his extensive studies on early amphibian morphogenesis, that certain pieces of head ectoderm and mesoderm, reared in isolation in salt solution (Holtfreter solution⁵) developed balancers, in some cases more than would normally have formed *in situ*. Discussion of the experimental results of our investigation will be reserved for future publication.

The technical details reported here readily fall into 3 topics and will be taken up in the order of their use in the experiments. The various technics to be discussed may well find application in many fields of investigation.

1. *Sterilization of tissue.* The cultivation of amphibian tissues, particularly those covered with epidermis, has always been hindered by the deleterious influence of the bacterial flora of the pond or tap water in which the animal or embryo is living. Larvae and adults are further contaminated by the passage into their immediate aqueous environment of their intestinal (bacterial) flora. In the past, this has limited the culture of amphibian surface and sub-surface tissues to short term studies or to the fortuitous occurrence of bacteria-free explants. Irradiation of amphibian tissue by ultraviolet light, with the aim of rendering tissues aseptic, always involves the danger of altering the tissue in some manner. The ideal method of rendering tissues sterile requires massive inhibition and death of contaminant bacteria, while the tissue to be studied is left in a normal or close to normal state.

The recent report of Detwiler, Copenhagen and Robinson⁶ on the increased survival of

¹ Fischer, A., *J. Exp. Med.*, 1922, 36, 393.

² Stöhr, P., Jr., *Arch. Entw. Mech.*, 1924, 102, 426.

³ Fell, H. B., *Proc. Roy. Soc. London, Ser. B*, 1934, 116, 316; *Phil. Trans. Roy. Soc. London, Ser. B*, 1939, 229, 407; *J. Roy. Microsc. Soc.*, 1940, 60, 95.

⁴ Holtfreter, J., *J. Exp. Zool.*, 1945, 99, 161.

⁵ Holtfreter, J., *J. Exp. Zool.*, 1943, 93, 251.

amphibian eggs and embryos, following massive operations, when kept in a solution of 0.5 to 1.0% sodium sulfadiazine is of great value to the experimental microsurgeon. We have been able to confirm this finding. However, Detwiler and his associates were concerned with essentially whole animals, which presumably are endowed with a certain amount of tolerance or resistance to bacterial attack. Such a situation does not prevail in the case of excised pieces of organs and tissues of these animals. Not only is the orderly integrative effect of the "whole" animal lost, but also the ratio of wound surface to mass is increased. When such pieces as limb buds are transferred to nutrient medium following treatment in 0.5-1.0% sodium sulfadiazine, the onset of bacterial growth is delayed somewhat. It is, however, not completely inhibited, and at the end of 48 hours necrosis and cytolysis of the explant ensues, the result of a very visible growth of bacteria. When the contaminated culture fluid was plated out on nutrient agar, a large number of colonies appeared in 48 hours. These were found to consist of gram-negative, motile rods and, on further test (lactose fermentation, indole formation, etc.), proved to belong to the *Escherichia coli* group. Any culture of amphibian tissues contaminated by such bacteria is doomed to rapid cytolysis.

It has been found that the addition of streptomycin in a concentration of 10-20 γ per milliliter to the sulfadiazine bath of Detwiler *et al.* will render small amphibians and their tissues completely aseptic in 24 hours in over 95% of attempts. The technic used in this laboratory is as follows: A 0.5% (for embryos) or 1.0% (for larvae) solution of sodium sulfadiazine is made up in tap water. To this is added streptomycin in a concentration of 10-20 γ /ml. This solution is placed in sterile covered stender dishes, in each of which one or two embryos or larvae are placed. The animals are kept in this solution for 24 hours at the end of which time they are sterile and ready for operation. They are anaesthe-

tized, if need be, in sterile MS 222.* The operative removal of the organ anlagen is carried out aseptically under a plexiglass shield mounted just below the oculars of a dissecting binocular microscope. Surgery is carried out using sterile iridectomy scissors, microscalpels, and watchmakers forceps. Each explant is removed from the operating dish with a mouth pipette, and placed in a bath of sterile Holtfreter solution. This bath is employed to promote the wound healing of the epidermis of the tissue piece, and to permit the diffusion from the tissue of the sulfadiazine and streptomycin solution. Explants are kept in these baths individually for a minimum of two hours. Following this, the tissue is removed from the bath with an absolute minimum of salt solution (again by mouth pipette) and transferred to a drop of nutrient on a sterile cover slip. The preparation is then sealed to a depression slide with a sterile paraffin-vaseline mixture. Experiments thus set up are placed in a constant temperature chamber at 20°C for further study.

The sulfadiazine-streptomycin solution here employed has exhibited no toxic effects whatever. Detwiler, Copenhaver and Robinson⁶ reported a retardation of growth of their animals when kept in 0.5-1.0% sodium sulfadiazine. Such an effect has not been noticed in our experiments. Both larvae and embryos continue to grow at normal rates, to undergo morphogenesis and to differentiate in a normal manner. Larvae and embryos have been kept for as long as one week in this solution with no visible ill effects. Young embryos (neurulae) may be removed from their capsules in this solution and develop normally.

The procedure outlined above does not inhibit the growth of fungi which may contaminate amphibia. However, if precautions are taken in the maintenance of the animals in the laboratory, fungus infection can be kept at a minimum (approx. 2%).

2. *Culture medium.* The medium used in

⁶ Detwiler, S. R., Copenhaver, W. M., and Robinson, C. O., *J. Exp. Zool.*, 1947, 106, 109.

* MS 222 $C_{10}H_{11}NO_2S$. A meta-amino-benzoic acid-ethylester in the form of a methan-sulfonate. (Sandoz Chemical Works, Basel and New York).

this study consisted of a mixture of two fluids: 1) Citrated rabbit plasma obtained by sterile heart puncture, and 2) a solution of half strength Holtfreter solution, containing as additions 0.25% agar, 0.045% sodium nucleate and 0.77% glucose. Equal portions of these two fluids were mixed to form the final medium. This medium has sustained the growth and differentiation of amphibian limb buds for as long as 20 days. Routinely, cultures are kept for from 4 to 12 days. For these periods it does not need to be replenished. This medium is not by any means considered to be perfect for amphibian tissue, nor is it felt that all requirements for growth and differentiation are supplied. It is hoped that further experiments will result in a medium in which all of the constituents are known chemically. The small amount of yolk included in embryonic explants undoubtedly constitutes a part of the essential nutrient. Tissues do survive, however, beyond the point where yolk is visible microscopically either in the culture or in the cells.

The above medium was developed after experiments had been made with the synthetic medium of White.⁷ It was found that White's medium alone would not support the growth of embryonic or larval amphibian tissue. Also, the inorganic salt solution of White, when used in place of Holtfreter solution in the above medium, would not support growth.

3. *Epidermal closure of the wound.* It has been found that the piece of tissue in culture must be covered by the epibolically active epidermis before organogenesis will take place. Holtfreter solution, as a bath for the explant prior to placing it in the hanging drop, serves well to enhance the wound healing capacities of the epidermis. In earlier stages this process may be observed under the low power

microscope. When healing of the epidermis does not take place, the explant forms a culture characterized by migrating cells and loss of structural organization. The medium as used also favors epithelial permanence, i.e., if the tissue is not in contact with the glass cover slip, but rather hangs down in the center of the drop. Epiboly of the epidermis is not hindered by the contact of the explant with the medium-air interface in the well of the slide. The agar contained in the medium tends to increase its viscosity to the consistency of a soft mush (Spratt⁸). The agar, plus the lack of fibrin strands to promote the outwandering of cells, is thought to assist in the maintenance and development of morphological organization of the explant within its epithelial sac.

By the use of the technics described above, it has been possible to study the differentiation *in vitro* of such complex organs and organ systems as limbs, hearts, gills, balancers, and eyes. Differentiation has been, in all cases where it occurred, essentially normal, although somewhat slower than in the intact controls.

Summary. 1. A technic is described for rendering surface and subsurface tissues of amphibia wholly sterile for use in tissue culture. Advantage is taken of the nontoxic selective antibiotic action of small concentrations of sodium sulfadiazine and streptomycin.

2. A medium which supports organogenesis, differentiation and growth of isolated organ and organ system primordia of amphibian embryos has been reported. The principal components of this medium are citrated plasma, salts, agar, glucose and sodium nucleate.

3. Morphogenetic activity in an explanted piece of tissue occurs only when the epidermis heals so as completely to cover the explant.

⁸ Spratt, N. T., Jr., *J. Exp. Zool.*, 1947, 100, 345.

⁷ White, P. R., *Growth*, 1946, 10, 231.

Value of Urea in the Diet of Rabbits.

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The rabbit shows some very interesting peculiarities in its nutrition in that it does not require a dietary source of riboflavin, pantothenic acid, biotin or folic acid.¹ On the basis of the excretion of these vitamins it is apparent that there is considerable microbial synthesis in the digestive tract of the rabbit. Rabbits show a normal functional coprophagy which presumably enables them to make better utilization of the vitamins synthesized in the digestive tract. It has been shown that more than 50% of the feces produced by the rabbit is normally reingested.²

The literature on the synthesis of the B vitamins and of protein by microorganisms in the paunch of ruminants has been previously reviewed.^{3,5} It is well established that elemental forms of nitrogen such as urea can be converted into protein by microorganisms in the rumen of polygastric animals and that the protein is utilized by the host animal. The fact that the rabbit has a large cecum favorable to microorganisms and the recent observations showing that they do not require a dietary source of some of the B vitamins prompted us to investigate the possibility of amino acid synthesis by the rabbit and its utilization during the normal process of feces ingestion. Observations have also been made in the course of these studies on the effect of decreasing the level of protein on the requirements for vitamin A.

Experimental. Weanling rabbits, 8 weeks old, of the New Zealand White breed and

weighing 800 g to 1200 g were used in these studies. There were 5 rabbits in each group with an equal number of males and females. These were kept in wire bottom cages. This type of cage does not prevent coprophagy in the rabbit. Group I was fed a diet composed of purified casein (Labco) 10%, DL-methionine 0.30%, cerelose 68.2%, cellulose 10%, Salts mixture⁶ 3%, corn oil 8%, A & D Oil (Nopco XX) 0.5%. The following amounts of vitamins were added per 100 g of diet: mixed tocopherols 50 mg, choline chloride 200 mg, niacin 20 mg, inositol 10 mg, pyridoxine hydrochloride 0.7 mg, thiamine hydrochloride 0.7 mg, riboflavin 0.7 mg, calcium pantothenate 1 mg, and 2-methyl, 1,4-naphthoquinone 0.075 mg. Group 2 was fed a diet similar to the above except that 3.43% of urea was added at the expense of an equal amount of dextrose to make the protein equivalent ($N \times 6.25$) to 20%. The third group received 20% of casein and no methionine. At the end of the 4th week the diets were slightly modified by replacing wood pulp for the cellophane as a source of cellulose. The methionine was added at levels so that the methionine content of the diets containing 10% of casein was equivalent to that of the diet containing 20% of casein. Since methionine has been shown to favor the synthesis of amino acids in ruminants⁷ it was added to eliminate this as a factor of variation with regards to the control group receiving 20% casein. Water and food were given *ad libitum*. The rabbits were kept on experiment for 10 weeks and weighed at weekly intervals.

The average growth response of the animals in each group is shown in Fig. I. As can be

¹ Olcese, Orlando, Pearson, P. B., and Schweigert, B. S., *J. Nutrition*, 1948, **38**, 577.

² Eden, A., *Nature*, 1940, **145**, 36.

³ Goss, Harold, *Nutrition Abstr. and Rev.*, 1943, **12**, 531.

⁴ McNaught, M. L., and Smith, J. B. A., *Nutrition Abstr. and Rev.*, 1947, **17**, 18.

⁵ Kon, S. K., and Porter, J. W. G., *Nutrition Abstr. and Rev.*, 1947, **17**, 31.

⁶ Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

⁷ Loosli, J. K., and Harris, L. E., *J. Anim. Sci.*, 1945, **4**, 435.

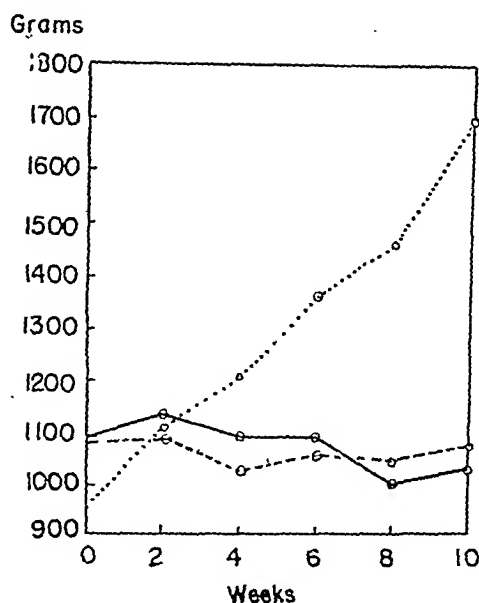


FIG. 1.

Growth of rabbits on diet containing 20% casein is indicated by dotted line. Broken line shows weight of rabbits receiving diet containing 10% casein and solid line weight of rabbits fed diet containing 10% casein plus 3.43% urea.

readily seen the group receiving 20% casein was the only group that made satisfactory gains. The groups receiving either the 10% casein alone or 10% casein plus 3.43% urea both lost weight. Since the rabbits receiving urea as a supplement to a low protein diet made no more gain than those on 10% of casein it is apparent that there is no significant synthesis of protein by microorganisms in the gastrointestinal tract of the rabbit.

At the end of the 3rd week 3 rabbits in each of the groups on the low protein diets showed some eye lesions. The main symptom was a conjunctivitis. A viscid conjunctival secretion was present and the lids were stuck together. Usually these symptoms became noticeable in both eyes at the same time. Oral administration of A and D oil for a period of one week relieved the animals of these early symptoms of vitamin A deficiency but when the administration was stopped the symptoms reappeared in one week. Again the A and D oil was administered for 1 week and the symptoms disappeared but they reappeared as soon as the administration of the

A and D oil was discontinued on the 7th week. None of the rabbits on the diet containing 20% casein showed vitamin A deficiency symptoms. Since the administration of the extra vitamin A did not influence the growth of the rabbits it may be assumed that the failure of the rabbits on the low protein diets to grow was due to a deficiency of protein *per se*. The fact that a vitamin A deficiency occurred with the rabbits fed low protein diets is very significant and suggests an interrelationship in the diet between proteins and fat soluble factors. This may be related to the preservation of vitamin A.

Although no carefully controlled experiments have been reported on vitamin A deficiency produced by the partial replacement of protein concentrates by urea, some contradictory evidence exists in the literature with regards to the effect of adding urea to silage. While Cullison⁸ has reported an increased content of carotene in silage which had received urea supplementation, Wise, *et al.*⁹ found losses of carotene in silage that was fed with urea as a supplement. On the other hand, Woodward and Shepherd¹⁰ have found no appreciable differences in the carotene content of silage containing urea and silage not containing urea.

Destruction of vitamin A can not be attributed to the addition of urea to the diet low in protein, because, although the rabbits fed this diet developed vitamin A deficiency symptoms, similar symptoms were shown by the rabbits fed the low protein diet that did not contain urea. The ill effects are therefore attributable to the lower protein level in the diet. Since it is very doubtful that the lower protein intake interferes with the absorption of fat-soluble factors, the mechanism by which the additional protein produces its beneficial effects is probably related to the synergistic effect of the amino acids in the stabilization of the fat. Clausen, Lundberg and Burr¹¹ have

⁸ Cullison, A. E., *J. Anim. Sci.*, 1914, **3**, 59.

⁹ Wise, G. H., Mitchell, J. H., La Master, J. P., and Roderick, D. B., *J. Dairy Sci.*, 1914, **27**, 649.

¹⁰ Woodward, T. E., and Shepherd, J. B., *J. Dairy Sci.*, 1914, **27**, 648.

¹¹ Clausen, D. F., Lundberg, W. O., and Burr, G. O., *J. Am. Oil Chem. Soc.*, 1917, **24**, 103.

shown that by the addition of adequate amino acid synergists the effectiveness of natural anti-oxidants, which are present in the fats in appreciable quantities, may be greatly increased. This stabilization of the fat may be an important factor in reducing vitamin A and carotene destruction.

Summary. The addition of urea to a diet low in protein did not increase the rate of gain of rabbits. From the data it is apparent that this species cannot use urea in the diet

as a replacement of part of the protein. Rabbits fed diets containing 10% of casein as the sole source of protein or 10% casein plus 3.43% urea developed typical vitamin A deficiency symptoms as compared with the control group fed a diet similar except that it contained 20% of casein. The results suggest that protein has a protective action on the oxidation of vitamin A in the diets or that it has a sparing action on the vitamin A requirements of the rabbit.

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Pantothenic Acid and the Metabolism of Amino Acids by Bacteria.*

R. C. KERSEY AND J. R. PORTER.

From the Department of Bacteriology, College of Medicine, State University of Iowa, Iowa City, Iowa.

Dorfman, Berkman and Koser,¹ and Hills² first observed that pantothenic acid functions in the pyruvic acid metabolism of *Proteus morganii*. More recently, Lipmann, *et al.*,³ and Novelli and Lipmann⁴ have found that pantothenate is a part of a coenzyme which is concerned with an acetylation system in tissues and with the breakdown of pyruvate to acetate and other products. They isolated this coenzyme, which they termed "coenzyme A", and were able to recover pantothenic acid from it. They also found that the amount of pantothenate bound in the cells closely correlated with the increased stimulation of pyruvate oxidation, and devised a method for the assay of coenzyme A.⁵

* This study was supported in part by a grant from the Williams-Waterman Fund for the combat of dietary diseases. Certain of the vitamins and amino acids were kindly supplied by Merek & Co.

¹ Dorfman, A., Berkman, S., and Koser, S. A., *J. Biol. Chem.*, 1942, **144**, 393.

² Hills, G. M., *Biochem. J.*, 1943, **37**, 418.

³ Lipmann, F., Kaplan, N. O., Novelli, G. D., Tuttle, L. C., and Guirard, B. M., *J. Biol. Chem.*, 1947, **167**, 869.

⁴ Novelli, G. D., and Lipmann, F., *Arch. Biochem.*, 1947, **14**, 23.

The present study was undertaken to determine if pantothenic acid has any effect on the metabolism of amino acids or other related compounds by bacteria.

Technic. The chemically-defined medium employed in this study for growing the bacteria has already been described.⁶ The organism used was *Proteus morganii*. Pantothenate deficient cells were prepared as follows: A small amount of growth was removed from a 6-hour agar slant culture by means of a sterile needle and cultured in 10 ml of a chemically-defined medium for 24 hours at 37°C. One ml of this culture was then used to inoculate larger quantities of the same basal medium containing in this case only 0.01 µg of Ca-pantothenate per ml. The "pantothenate-deficient" cells from 4 liters of a 24-hour culture were then harvested by means of a Sharples centrifuge, and washed twice with 50 ml of sterile saline by the ordinary centrifuge technics. From this quantity of medium it was possible to obtain yields of cells ranging in dry weight from 150

⁵ Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, 1948, **174**, 37.

⁶ Pelezar, M. J., Jr., and Porter, J. R., *J. Biol. Chem.*, 1941, **139**, 111.

TABLE I.

Effect of Pantothenate on Ammonia Liberation and on Oxidation of Amino Acids by *Proteus morganii*.

Amino acid	$\mu\text{g NH}_3$ produced in 90 min.*		$\mu\text{l O}_2$ -uptake in 90 min.*	
	Without pantothenate	With pantothenate	Without pantothenate	With pantothenate ^a
Glycine	10	12	3	5
dl-Alanine	13	23	7	10
dl-Valine	7	16	22	26
dl-Threonine	30	43	8	17
dl-Phenylalanine	5	8	0	5
dl-Methionine	14	14	0	6
dl-Isoleucine	13	20	25	29
dl-Citrulline	0	2	13	15
l-Tryptophane	4	6	0	0
l-Proline	5	5	3	5
dl-Norleucine	20	25	6	13
dl-Lysine, HCl	2	4	6	8
l-Histidine, HCl	4	6	0	5
l-Cystine	11	15	15	25
d-Arginine, HCl	5	8	13	19
dl-Tyrosine	8	14	8	10
dl-Serine	74	79	85	94
dl-Norvaline	27	35	13	14
l-Hydroxyproline	0	0	0	4
dl-Glutamic acid	20	48	23	172
dl-Aspartic acid	16	30	12	50
dl-Leucine	30	38	12	13

* Average values for several determinations; all data corrected for controls which were 3.5 μg of NH_3 and 10-15 $\mu\text{l O}_2$.

to 200 mg. After the final washing and centrifugation the cells were resuspended in saline to give a final concentration of 10 mg of cells per ml. The substrates employed in this study consisted of the 22 amino acids and certain organic acids shown in the tables. These compounds were all prepared in concentrated aqueous solutions, and used as M/450 final concentrations.

For the deamination studies the micro-diffusion method of Conway⁷ was employed, and the ammonia determinations were made as follows: The substrates (amino acids) plus pantothenate were added to the outside well of the diffusion cell, together with phosphate buffer (pH 8.0) and a 5 mg suspension of bacteria, to give a final quantity of 1.5 ml. One ml of N/150 HCl, containing an indicator, was placed in the center cup of the diffusion cell. Following an incubation period of 90 minutes at 37°C, 1 ml of saturated K_2CO_3 solution was added to the outside well

to release the ammonia, and the units were again incubated for one hour. The acid in the center cup was then titrated with N/75 NaOH, and the amount of ammonia liberated from the amino acids calculated.

For the respiration studies, standard manometric methods were employed. The substrates were added so as to give M 450 final concentrations: 0.1 ml of N 10 HCl was placed in the side bulb to absorb any ammonia given off by the 1.0 ml bacterial cell suspensions; and 0.3 ml of a 20% KOH solution was placed in the center well of the Warburg vessels. In every case the final volume of the vessels was brought to 3.0 ml with phosphate buffer (pH 7.3). The flasks were then equilibrated for 10 minutes in the water bath before readings were started.

Experimental and results. From the data in Table I it will be seen that serine was the most readily deaminated amino acid. However, since this reaction was not stimulated by pantothenate it does not appear to be involved in the process. Alanine, threonine, norleucine, norvaline, glutamic acid, aspartic

⁷ Conway, E. J., *Micro-diffusion Methods and Volumetric Error*, 1940, D. Van Nostrand Co., New York.

acid, and leucine were also deaminated in varying degrees, and in certain of these cases the presence of pantothenate appeared to stimulate the process. This was especially true in the case of glutamic and aspartic acids. Few of the other amino acids were attacked by this organism, nor did the presence of pantothenate stimulate ammonia liberation.

Since it is generally accepted that deamination is one of the first steps in the breakdown of amino acids, especially at the pH values where the above experiments were conducted, an attempt was made to determine the role played by pantothenate in the metabolism of certain of these substances. It was found after a few preliminary experiments that the respiration of "pantothenate-deficient" cells of *Proteus morganii*, using amino acids as substrates, showed a definite lag phase during the first half hour or so of the experiment. Since it has been reported by McIlwain⁸ that a time factor is involved when deficient cells bind pantothenate, it was decided to divide the cell suspensions into two equal portions after the final washing, and add 1.0 μ g per ml of pantothenate to one portion of the cells. When the cells were treated in this manner, followed by incubation at 37°C for about 2 hours, little or no lag was noted in their early respiration period.

From the oxygen uptake values (Table I) it will be seen that serine gave the highest values for deficient cells and like-

TABLE II.
Effect of Pantothenate on Oxidation of Glutamic Acid, Aspartic Acid, and Their Metabolic Intermediates.

	μ l O ₂ uptake in 90 min.*	
	Without pantothenate	With pantothenate
Glutamic acid	20	170
α Ketoglutarate	16	143
Succinate	44	103
Fumarate	10	23
Oxalacetate	20	55
Pyruvate	30	64
Aspartic acid	15	55

* Average values for 4 determinations; all data corrected for controls which gave 5-15 μ l O₂ uptake.

⁸ McIlwain, H., *Biochem. J.*, 1945, 39, 279.

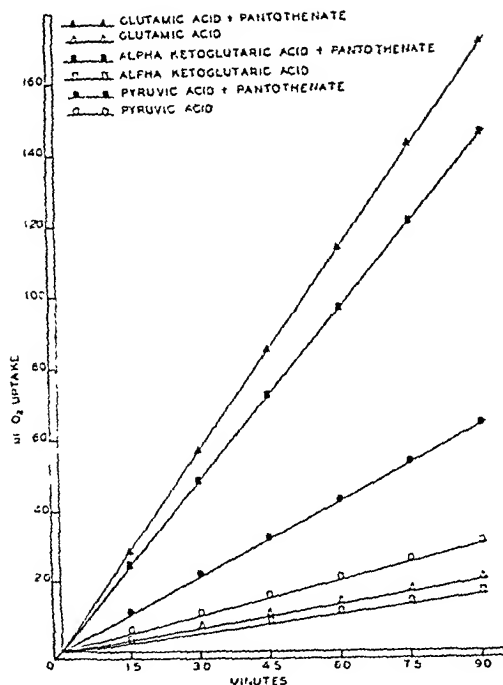


FIG. 1.

Effect of pantothenate on oxidation of glutamic acid, α -ketoglutarate, and pyruvate.

wise quite high values for non-deficient cells. As in the case of the deamination studies, however, pantothenate did not stimulate the oxidation of serine. On the other hand, when such amino acids as threonine, norleucine, cystine, and especially glutamic and aspartic acids were used as substrates, pantothenate did stimulate their oxidation in varying degrees. The fact that the oxidation of these amino acids is stimulated by pantothenate would seem to indicate that pyruvate, which is known to be stimulated, is the key intermediate in their metabolism. With this in mind glutamic acid and aspartic acid were chosen as representative amino acids for further metabolic studies. In these experiments glutamic acid and aspartic acid were studied together with their known, or postulated, intermediates in an attempt to discover at which point, or points, in their metabolism pantothenic acid functions. The intermediates studied were α -ketoglutarate, succinate, fumarate, oxalacetate and pyruvate, and these were kept on an equal molar basis

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dl-Citrulline	0	2	13	15
l-Tryptophane	4	6	0	0
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dl-Norleucine	20	25	6	13
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⁷ Conway, E. J., *Micro-diffusion Methods and Volumetric Error*, 1940, D. Van Nostrand Co., New York.

mine the effect of 12 cm electromagnetic radiations of testicular tissue. In addition, attempts were made to confirm the results of previous investigators relative to the effects of infra red irradiations on testes.

Procedure. Male albino rats of the Sprague-Dawley strain, ranging in age from 120 to 200 days were employed in this study. In preparation for irradiation the animals were anesthetized with ether and the scrotum swabbed with 95% alcohol. Each animal was then arranged on a platform behind a copper shield, and the scrotum was inserted through an opening provided in the shield. Thus, the remainder of the animal was protected from the radiations. An iron-constantan thermocouple needle, of the type described by Tuttle and Janney,⁵ was then inserted into the center of one of the testes. This served to register the degree of temperature produced. The thermocouple had been calibrated previously with a Bureau of Standards thermometer. Thermocouple potentials were measured by a Leeds and Northrup potentiometer. Temperatures were read to the nearest tenth of a degree centigrade. The temperature of the testes was allowed to fall to approximately 29°C before the irradiations were started. Because of possible damage to the testes resulting from thermocouple needle puncture, one testis was employed for temperature measurements and the contralateral testis was used to study the histologic effects of the radiation. Care was taken to align both testes at an equal distance from the source of radiation. In preliminary experiments it was found that if these precautions were taken the temperature rise due to the radiations was the same in both testes.

A Raytheon Microtherm generator (model CMD4) which produced a wave length of approximately 12 cm was used to apply the high frequency radiations. A variac was provided by means of which the power output was regulated. The corner type reflector was used. The infra-red source rated at 600 watts was of the non-luminous type, and a 9-inch hemispherical reflector was employed. In all

cases irradiation was applied to the testis through the scrotum.

Two series of experiments were performed with 12 cm electromagnetic waves. In the first of these series the procedure consisted of elevating the testicular temperature to levels of 47, 46, 45, 44, 43, 40, 37, 35, 34, 33, 32, 31 and 30°C. For each temperature level 4 animals were used, and the testicular temperature was maintained at the selected temperature for a single period of 5 minutes. The temperature was maintained by varying the power output of the microwave machine. From each group of 4, an animal was sacrificed at 4, 8, 12 and 16 days following the exposure and the testes were prepared for histologic examination.

In the second series of irradiations 2 groups of 10 animals were given single 15-minute exposures at temperature levels of 33° and 34°C. A third group of 10 animals was exposed to 35° C for a period of 10 minutes. All animals were sacrificed for histologic studies 4 days following the exposures. The testes were fixed in Bouin's solution and the sections stained in hematoxylin-eosin.

Infra-red irradiation was used in two series of experiments. In the first series testicular temperatures were raised by irradiation to levels of 33, 37, 40, and 43°C for periods of 5 minutes. Four animals were exposed at each temperature level and a histologic examination of the testes was made at 4, 8, 12, and 16 days after the exposures. The procedure in the second series of infra-red irradiations was to expose two groups of 10 animals to temperature levels of 38° and 40°C for 10 minutes. Four days after exposure the animals were sacrificed and the testes were removed for histologic study.

The testes of 12 normal non-irradiated males were employed as histologic controls.

Results. The results of experiments with 12 cm electromagnetic irradiations are listed in Table I. These results show that in all cases when the testicular temperature was raised to 35°C or higher there was evidence of testis tissue damage. At temperatures from 31° to 35°C approximately 50% of the testes showed signs of degenerative changes. The

⁵ Tuttle, W. W., and Janney, C. D., *Arch. Phys. Med.*, 1948, 29, 416.

with glutamic acid. The results of these studies are shown in Table II and Fig. 1. From these data it can be seen that pantothenate stimulated respiration considerably when either α -ketoglutarate or glutamic acid was used as a substrate, but succinate, fumarate, oxalacetate and aspartic acid were oxidized to lesser extent. It will be noted that the stimulation values for most of these substrates are in the same range as was observed with pyruvate. It was also possible to demonstrate pyruvate in the vessels after all of these substrates had been oxidized. Thus it can probably be assumed that pantothenate stimulation of certain amino acids and other substrates is due, in part at least, to their being converted to pyruvate which is in turn stimulated. However, the pantothenate stimulation observed when glutamic acid or α -

ketoglutarate was used as substrate cannot be accounted for entirely as being due to pyruvate stimulation alone. Therefore it appears that pantothenic acid also functions in some other way in the oxidation of these substrates. Since the values observed with glutamic acid were very close to those noted with α -ketoglutarate, it would seem to eliminate the possibility of pantothenic acid functioning in the deamination of glutamic acid.

Summary. From this study it can be concluded that the pantothenate stimulation of amino acid oxidation by *Proteus morganii* is limited to those amino acids which can be converted to pyruvate. However, in the case of glutamic acid, and its metabolic intermediate α -ketoglutarate, the pantothenate stimulation appears to be more than can be attributed to pyruvate oxidation.

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Testicular Degeneration as a Result of Microwave Irradiation.

C. J. IMIG, J. D. THOMSON, AND H. M. HINES.

From the Department of Physiology, State University of Iowa.

The mammalian scrotum has been established as being a local thermoregulator for the testes. Moore and Quick¹ found the scrotal temperature of white rats to be from 2° to 8°C lower than the temperature of the abdominal cavity. A sub-abdominal temperature in the scrotum has been shown to be necessary for the continuance of spermatogenesis. Moore² confined the testes of guinea pigs in the abdominal cavity for varying periods of time and found that an abdominal retention of seven days resulted in a complete disorganization of the germinal epithelium of the seminiferous tubules. He considered the cause of this degeneration to be due to the higher temperature of the abdominal cavity.

Investigations of Moore³ showed that testic-

ular degeneration resulted from a single application of heat at approximately 7°C above body temperature for a 15-minute period. The heating devices which were used consisted of hot water baths, electric stoves, electric light bulbs, and hot water pads. Testicular degeneration was visible histologically within four to six days following the heat application, and was entirely similar in type to that resulting from early experimental cryptorchidism.

Fukui⁴ exposed the scrotum of rabbits to sunlight and to warm air, and found a definite relationship between the temperature and the time required to cause regressive changes in the germinal cells. The minimum scrotal temperature at which he was able to produce testicular damage was 40°C. The time of required exposure at this temperature was more than one hundred hours.

It was the purpose of this study to deter-

¹ Moore, C. R., and Quick, W. J., *Am. J. Physiol.*, 1924, 68, 70.

² Moore, C. R., *Am. J. Anat.*, 1924, 34, 269.

³ Moore, C. R., *Am. J. Anat.*, 1924, 34, 337.

⁴ Fukui, N., *Japan M. World*, 1923, 3, 27.

mine the effect of 12 cm electromagnetic radiations of testicular tissue. In addition, attempts were made to confirm the results of previous investigators relative to the effects of infra red irradiations on testes.

Procedure. Male albino rats of the Sprague-Dawley strain, ranging in age from 120 to 200 days were employed in this study. In preparation for irradiation the animals were anesthetized with ether and the scrotum swabbed with 95% alcohol. Each animal was then arranged on a platform behind a copper shield, and the scrotum was inserted through an opening provided in the shield. Thus, the remainder of the animal was protected from the radiations. An iron-constantan thermocouple needle, of the type described by Tuttle and Janney,⁵ was then inserted into the center of one of the testes. This served to register the degree of temperature produced. The thermocouple had been calibrated previously with a Bureau of Standards thermometer. Thermocouple potentials were measured by a Leeds and Northrup potentiometer. Temperatures were read to the nearest tenth of a degree centigrade. The temperature of the testes was allowed to fall to approximately 29°C before the irradiations were started. Because of possible damage to the testes resulting from thermocouple needle puncture, one testis was employed for temperature measurements and the contralateral testis was used to study the histologic effects of the radiation. Care was taken to align both testes at an equal distance from the source of radiation. In preliminary experiments it was found that if these precautions were taken the temperature rise due to the radiations was the same in both testes.

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Two series of experiments were performed with 12 cm electromagnetic waves. In the first of these series the procedure consisted of elevating the testicular temperature to levels of 47, 46, 45, 44, 43, 40, 37, 35, 34, 33, 32, 31 and 30°C. For each temperature level 4 animals were used, and the testicular temperature was maintained at the selected temperature for a single period of 5 minutes. The temperature was maintained by varying the power output of the microwave machine. From each group of 4, an animal was sacrificed at 4, 8, 12 and 16 days following the exposure and the testes were prepared for histologic examination.

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Results. The results of experiments with 12 cm electromagnetic irradiations are listed in Table I. These results show that in all cases when the testicular temperature was raised to 35°C or higher there was evidence of testis tissue damage. At temperatures from 31° to 35°C approximately 50% of the testes showed signs of degenerative changes. The

⁵ Tuttle, W. W., and Janney, C. D., *Arch. Phys. Med.*, 1948, 29, 416.

TESTICULAR DEGENERATION

TABLE I.
Effect of 12 Cm Irradiation on Male Gonads.

No. animals exposed	Exposure temp. °C	Time of exposure min.	Animals with testicular damage	% with testicular damage
4	47	5	4	100
4	46	5	4	100
4	45	5	4	100
4	44	5	4	100
4	43	5	4	100
4	40	5	4	100
4	37	5	4	100
4	35	5	4	100
4	33	5	2	50
4	32	5	1	25
4	31	5	1	25
4	30	5	0	0
13	35	10	13	100
4	33	10	1	25
4	31	10	1	25
3	35	15	3	100
7	34	15	3	43
16	33	15	9	56
4	31	15	0	0

TABLE II.
Effect of Infra Red Irradiation on Male Gonads.

No. animals exposed	Exposure temp. °C	Time of exposure min.	Animals having testicular damage	% with testicular damage
3	43	5	2	67
4	40	5	2	50
4	37	5	0	0
4	33	5	0	0
8	40	10	3	38
10	38	10	0	0

testes of animals exposed to 30°C were not affected.

The results of experiments with infra red irradiation (Table II) show that testes from 67% of the animals exposed to 43°C were damaged. Testicular degeneration was not found in the experiments where the temperature of the testes was maintained at 38°C for 10 minutes by infra red. No damage was found in the testes of control animals.

A typical histological picture of testicular degeneration following exposure to electromagnetic waves shows an area of degeneration along the side of the testis nearest to the source of radiation (area A, Fig. 1). Continuing from this area of degeneration to the opposite side of the testis, all gradations from completely degenerated to normal tubules can be found (areas B and C, Fig. 1).

Fig. 2 clearly shows the transition of degeneration from area A to area B of Fig. 1. These areas are well defined. A detailed picture of a degenerating tubule in area B is shown in Fig. 3. In area A, which was nearest to the source of radiation, complete coagulation of the tubules was found (area A of Fig. 2). This area showed an absence of germ cell nuclear material, the cytoplasm appearing much more refractile than normal cytoplasm. The tissue resembled that seen in burn necrosis. In general the tubules in area B show sloughing of degenerating germinal elements into the lumen, multinuclear masses termed "giant cells" which apparently consist of fused spermatid nuclei, and usually absence of spermatozoa (area B of Fig. 2 and 3). Sertoli and interstitial cells apparently remain intact. Leucocytic infiltration of the

TESTICULAR DEGENERATION

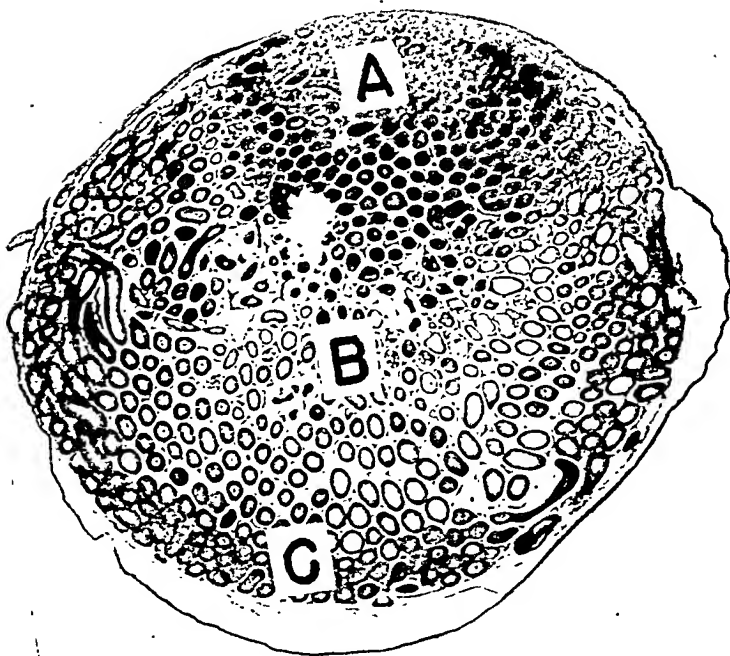


FIG. 1.
Cross-section of entire testis removed 4 days after a single irradiation with 12 cm microwaves at 35°C for 10 minutes ($\times 10$). See text for explanation of the areas indicated.



FIG. 2.
Seminiferous tubules from areas A and B of Fig. 1. ($\times 40$).



FIG. 3.
High magnification of a degenerating seminiferous tubule from area B of Fig. 2. ($\times 200$).

tissue was evidenced by the presence in some cases of intertubular polymorphonuclear leucocytes, in the areas of degeneration. In area C of Fig. 1, the tubules were essentially normal.

Fig. 4 is a cross section through the center of a testis exposed for 10 minutes at 40° C to infra red irradiations. Area A, the side most directly exposed, shows coagulation of the tubules, whereas the tubules in area B show varying degrees of degeneration. The tubules farthest from the source of radiation were normal. The general picture of the degenerative changes was similar to that produced by microwave irradiation.

Discussion. Testicular degeneration resulting from exposures to microwaves and infra red irradiations presented a similar histologic appearance which was typical of the degeneration seen in experimental cryptorchidism.

The temperatures at which damage was noted from infra red irradiations were approximately 3 to 5°C lower than those reported by Moore and Chase. These investigators placed the bulb of a thermometer close to the scrotum to register the degree of heat applied. With the needle thermocouple

method of temperature measurement used in the experiments reported here it was possible to register the temperature within the testes.

Following electromagnetic irradiation testicular degeneration was found at temperatures below those at which damage occurred from infra red irradiations. All of the testes which were elevated to a temperature level of 35°C and above with microwaves were found to contain degenerated tubules.

The outcome of this experiment clearly shows that testicular damage will result from 12 cm irradiations at a temperature below that of the abdominal cavity and below that necessary to cause injury by infra red exposures. This finding suggests that damage may result in part from factors other than heat. However, it should be pointed out that measurements of temperature were made only near the center of the testes and the possibility exists that areas adjacent to the field of irradiation may have been subjected to temperatures somewhat in excess of those recorded.

These findings suggest that precautions should be taken by those working in the field of high frequency electromagnetic generators and to those giving treatments with microwave generators. Because of the unusual susceptibility of testicular tissue to thermal agents, it seems desirable to shield these structures from high frequency electromagnetic waves during periods of treatment or exposure.

Summary. A study was made concerning the effects of 12 cm electromagnetic waves and of infra red irradiations upon the testes of adult albino rats. A single ten-minute exposure to microwaves at a temperature of 35°C as measured in the central areas of the testes caused testicular degeneration in all cases. In some experiments testicular damage resulted from a single exposure at temperatures between 30° and 35°C. Testicular damage was not found in experiments in which infra red irradiation was applied for 10 minutes at 38°C but was observed when applied at a temperature of 40° C and above. The type of degeneration resulting from microwave exposure could not be distinguished from that produced by infra red.

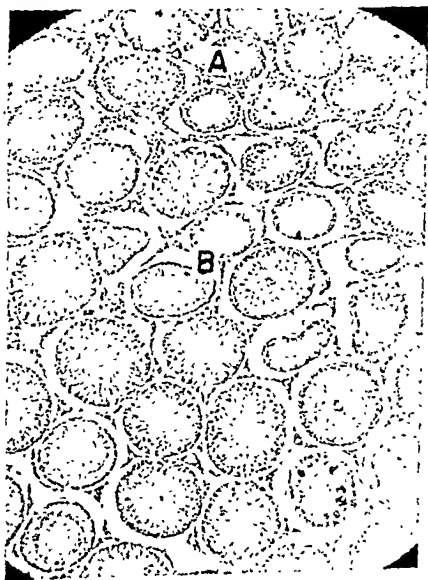


Fig. 4.

Degenerating tubules of a testis 4 days after a single irradiation with infra red at 40°C for 10 minutes. ($\times 40$).

Intravenous Infusions of a Combined Fat Emulsion into Dogs.*

B. G. P. SHAFIROFF, H. BARON, AND E. ROTH.

From the Laboratory of Experimental Surgery, Department of Surgery, New York University College of Medicine.

The availability of an intravenous emulsion of fat, protein and glucose for experimental and clinical investigation has opened new fields of study in parenteral nutrition.¹ A combined emulsion such as developed in our laboratory was administered intravenously with equal facility to human patients² as well as to animals with a favorably low incidence of infusion reactions. The present report is devoted to observations made on a series of dog recipients of the combined emulsion.

Method of study. Nineteen dogs were divided into 3 groups: Group I, seven dogs received single infusions of the combined emulsion in a variety of acute experiments. Group II, six dogs were maintained on daily infusions of the emulsion and a standard kennel diet. Group III, six dogs were carried exclusively on the intravenous emulsion as the sole nutritive source.

Two types of combined emulsion were used. The first type contained 5% coconut oil, 5% amigen and 5% glucose solution emulsified with a 6% special intravenous gelatin solution. The second type of combined emulsion contained 10% coconut oil with the remaining ingredients at the original concentrations. The caloric value of the 5% emulsion was 0.85 calories per ml, that of the 10% emulsion was 1.3 calories per ml. The emulsion was prepared in a special homogenizer constructed to our specifications so that the entire process of blending and homogenization could be carried out under sterile conditions and the resultant yield collected in

sterile bottles. The use of the autoclave as a final step in the procedure was obviated. The process of manufacture and a description of the homogenizing machine were described elsewhere in greater detail.^{1,2}

The following laboratory tests were made, the number of tests performed depending on the experiment; hemoglobin (Sahli), red blood cell count, white blood cell count, refractive index of plasma (Fischer refractometer), specific gravity of blood and plasma (Scudder apparatus) chylomicra count,³ total serum lipids and serum lipase.¹ The weight of the animals was recorded at regular intervals. After the completion of an experiment the dog was sacrificed and selected tissues were stained with special fat dyes for microscopic study.

Results. Group I: Three dogs were anesthetized with sodium pentobarbital, the femoral artery was cannulized and connected to a recording manometer. The blood pressure was continuously recorded throughout the infusion and for 2 hours thereafter. In all three of the dogs there was an elevation of 15 to 18% in blood pressure above the initial level. This occurred after $\frac{3}{4}$ of the total amount of emulsion had been infused. In 2 dogs the thoracic duct was intubated with a Lindemann needle and samples of lymph were tapped at regular intervals. The flow of lymph increased about 22% during the infusion period. The specific gravity of the lymph and refractometric examinations showed little change indicative of the presence or appearance of the emulsion into the lymph circulation. In each of 2 dogs the effect of rapid and slow infusion of the emulsion was tested.

* This project was made possible by grants from the Ben Lewis Fund in Experimental Surgery.

¹ Shafiroff, B. G. P., and Frank, C., *Science*, 1947, 100, 474.

² Unpublished.

³ Frazer, A. C., and Stewart, H. C., *J. Physiol.*, 1939, 95, 21.

⁴ Lagerlof, H., *Acta med. scandinav.*, 1945, 102, 407.

TABLE 1.
Effect of Infusions of Combined Emulsion.

Dog No.	Group	Days	Total g fat infused	Wt (kg)	Hemoglobin (g %)	Red blood cell count (million)	Hematocrit (Wintrube)	Plasma protein (g %)
9	11-5% combined emulsion 250 ml + diet	1	12.5	14.3	12.9	5.73	45	5.62
		7	87.5	14.3	12.9	6.62	45	5.80
		15	187.5	15.0	13.1	5.62	43	5.78
		21	262.5	15.6	13.9	6.50	45	6.21
		30	375	15.8	13.9	6.28	49	6.17
11	11-5% combined emulsion 500 ml + diet	1	95	11.0	10.2	4.52	51	4.96
		7	175	11.0	12.9	5.81	49	5.48
		15	375	11.2	12.2	6.26	44	5.23
		21	525	11.3	13.2	6.08	48	5.44
		30	750	11.8	12.9	6.00	49	5.35
12	11-5% combined emulsion 500 ml + diet	1	50	16.8	15.3	5.90	51	6.26
		8	400	16.3	15.0	5.80	51	6.00
		15	750	16.6	15.0	5.84	50	6.31
14	11-10% combined emulsion no food	1	50	16.5	14.5	6.18	49	5.92
		8	400	16.0	14.9	6.20	50	5.92
		15	750	15.5	15.2	6.10	48	6.00
19	11-10% combined emulsion no food	1	50	17.0	15.5	6.62	52	6.78
		8	400	17.2	16.0	6.58	51	6.90
		15	750	17.0	15.2	6.25	53	6.62



FIG. 1.

Section through the lung of a dog from Group III maintained exclusively on the 10% combined emulsion for a period of 14 days. The lung is microscopically normal.

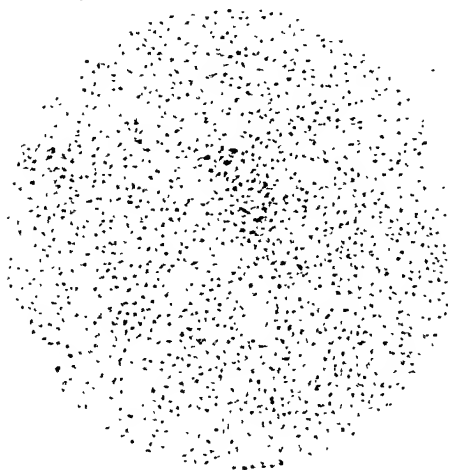


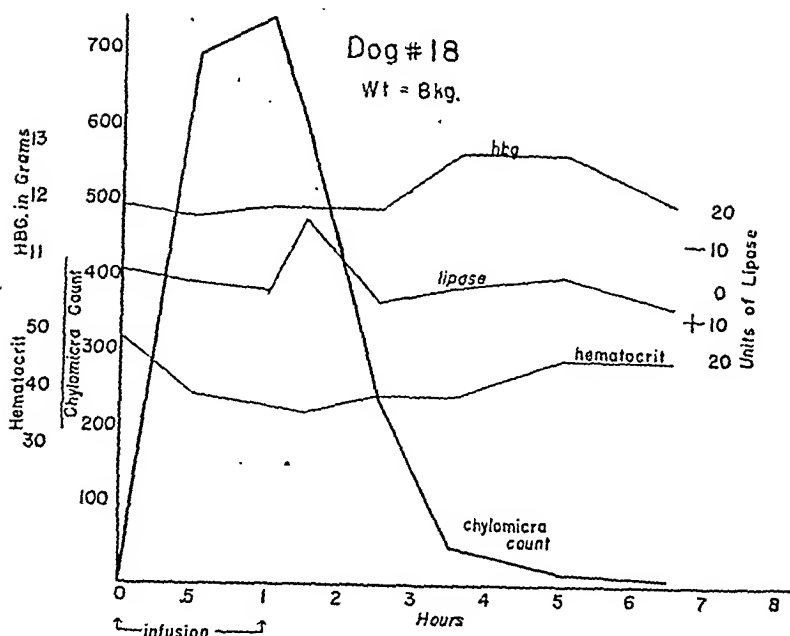
FIG. 2.

Section through the liver of the same dog. Liver shows areas of focal accumulations of fat still to be metabolized. The liver architecture is normal.

One dog received 500 ml of the emulsion within 90 minutes, the other received an equal volume over a period of 300 minutes. Both dogs tolerated the emulsion well. Plasma samples obtained during the rapid infusion were milky white in color and the emulsion could be readily detected. On nephelo-

metric examination there was a marked increase in the suspension concentration of such samples over that of the initial plasma sample. In the former samples a "cream layer" was obtained on standing in a narrow tube or after high speed centrifugation. All plasma samples obtained during the slow infusion were clear in color and showed little turbidity as determined by the nephelometer. The rate of disappearance of the emulsion from the blood as determined by the total serum lipids and the chylomicra count differed from that of the physical disappearance of the emulsion from the plasma. Depending on the rate of infusion there was a progressive rise in the chylomicra count and the total serum lipids and within 4 hours after completion of the injection both latter values returned to the level of their fasting state in the blood.

Group II. Four dogs in this group received daily infusions of the 5% combined emulsion for a 30 day period. They were allowed in addition a standard kennel diet. Dog No. 8 was injected with 150 ml of the emulsion, dog No. 9 with 250 ml, dog No. 10 with 400 ml and dog No. 11 with 500 ml of the emulsion. The number of injected calories ranged from 127 to 425 calories daily. Dog No. 8 received a minimum of 225 g of injected fat while dog No. 11 received the maximum of 750 g of fat during the study period. Two other dogs in this group No. 12 and No. 13 were infused daily with 500 ml of the 10% combined emulsion for a period of 14 days receiving also a total of 750 g of fat. In the dogs infused with large amounts of fat there was a corresponding decrease in appetite for the kennel diet. On many occasions the dogs refused the kennel diet entirely and accepted water only which was taken in large quantities. The dogs were always alert and energetic and never offered resistance throughout the infusion phase. During the injection there was a marked elevation in the chylomicra count in the blood followed by a fall to the pre-infusion level within four hours of completion of the infusion. Lipase activity determinations run concurrently with the latter revealed a significant increase in enzymatic activity in the blood. Red blood counts and



GRAPH I.

Typical graph of the chylomicra count, hemoglobin, lipase activity and hematocrit obtained in Dog No. 18 (Group III) on the 10th study day during the infusion and post-infusion phases of an injection of 500 ml of the 10% combined emulsion.

hemoglobin determinations made at regular intervals during the 30 day period showed no signs of hemolytic anemia. The weight gain in the animals varied from 0.8 kg to 1.6 kg. The largest weight gain occurred in dogs No. 8 and No. 9 who received the smallest volume of emulsion and ate more of the kennel diet than the others of the group. Examination of the tissues revealed the liver cells to contain intra-cellular fat with an occasional focal aggregation of lymphocytes. No true granulomata were observed in the lung tissue. The architecture of the kidney and spleen was normal.

Group III. All the dogs of this group were maintained exclusively on daily infusions of 500 ml of the 10% combined emulsion for a period of 14 days. The total amount of fat injected was 750 g. The dogs were allowed water ad libitum, received orally multiple high potency vitamins and were rewarded after each infusion with 2 rye-krisp biscuits. Chylomicra counts showed the characteristic tendency to increase during the infusion and

for a short period thereafter. Lipase activity increased in the blood during the infusion. The specific gravity of the blood and the plasma proteins stayed approximately stationary during the experimental period. Hemoglobin determinations and the red cell counts showed little variations from the original and secondary anemia did not develop. In this group dogs No. 14, 17 and 18 lost one-half to one kilogram in weight while dogs No. 15, 16 and 19 maintained their original weight with slight variations. Tissue studies in this group were also negative.

Comments. The utilization of intravenous fat emulsion for growth and energy has been effectively proved in dogs by many workers.^{5,6} The present experiments have demonstrated that the combined emulsion containing 5 or 10% fat may be used intravenously both as a supplement to diet or as the sole source of

⁵ McKibbin, J. M., Ferry, R. M., and Stare, F. J., *J. Clin. Invest.*, 1946, **25**, 679.

⁶ McKibbin, J. M., Hegsted, D. M., and Stare, F. J., *Fed. Proc.*, 1943, **2**, 9.

provision of the minimal daily requirements of the animal. Although mild to serious anemias have been reported to develop as a result of the infusions of the fat emulsions^{7,8} the latter complication failed to occur in the present investigation. This is believed to be due to the fact that phosphatide stabilizers were not used in the preparation of our emulsion. The disappearance of fat from the circulating blood determined either by the chylomicra count or by total serum lipids was interpreted to indicate that lipolysis commenced in the blood, the process being initiated by serum pancreatic lipase. Evidence to support the latter view was based on the increase of lipase activity of the blood and substantiated further by the report of Meng and Freeman⁹ that the fatty acids increased during the infusion of a fat emulsion. The assimilation of preformed fat infused intravenously may therefore be conceived as a possible biochemical function whereby the

neutral fat ultimately reached the liver for catabolism. An analysis of the slides revealed that in the acute experiments after an infusion all the organs were richly laden with fat. In the lung the fat did not permeate into the alveolar spaces but was contained in the capillaries. In the chronic experiments of Group II and Group III, the liver and the spleen were the only organs which contained evidences of fat.

Summary. (1) A combined emulsion of fat protein and glucose was infused intravenously in a series of 19 dogs.

(2). Toxic reactions such as secondary anemia and degenerative pathology in vital organs failed to develop after prolonged injections of the combined emulsion.

(3). An attempt was made to correlate the associated laboratory tests with the biochemical functions of hydrolysis and assimilation of fat.

The authors wish to acknowledge the helpful advice of Professors Mulholland and Co Tui. The authors are indebted to the E. F. Drew Co., to the Mead Johnson Co., and to the C. F. Knox Co. for generous supplies of material used for the preparation of the emulsion. The homogenizer was made by the C. W. Logeman Co., New York City.

⁷ Johnson, V., Longini, J., and Freeman, L. W., *Science*, 1943, 97, 400.

⁸ Dunham, L. J., and Brunschwig, A., *Arch. Surg.*, 1944, 48, 395.

⁹ Meng, H. C., and Freeman, S. J. *Lab. and Clin. Med.*, 1948, 33, 689.

16731

Effect of Alloxan Diabetes on Hyaluronidase Level of the Rat Testis.*

MAXINE MOORE. (Introduced by S. L. Leonard.)

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Although hyaluronidase, an enzyme obtained from mammalian sperm and testes, can disperse follicle cells of ovulated mammalian ova, a normal role of this enzyme in

* Aided by a grant from the Sage Fund of the Cornell University Trustee-Faculty Committee on Research, administered by Dr. S. L. Leonard.

¹ Chang, M. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, 60, 51.

² Leonard, S. L., Perlman, P. L., and Kurzrok, R., *Proc. Soc. Exp. Biol. and Med.*, 1947, 60, 517.

reproduction remains to be established.^{1,2} In line with the main problem of determining this role it was desired to learn if a physiological disturbance of the metabolism of the animal, without the destruction of the germinal elements, could alter the level of hyaluronidase within the testis. Experimental diabetes, therefore, was induced in male rats and after one month the amount of hyaluronidase was measured in the control and diabetic rat testes.

TABLE I.
Hyaluronidase Concentration in Normal and Diabetic Rat Testis. (Turbidimetric Method).

Testes	Normal		Diabetic		Significance of Diff. of means
	No. of rats	TRU/g*	No. of rats	TRU/g	
Chilled, homogenate	10	10.1 \pm 1.5†	10	10.4 \pm 2.8	P = .90
Incubated, homogenate	9	46.6 \pm 6.6	9	52.2 \pm 17.4	P = .75
+ Sem. ves. incubated	5	68.1 \pm 3.8	5	68.6 \pm 4.3	P = .90

* TRU: turbidity reducing unit.

† Standard deviation.

TABLE II.
Hyaluronidase Concentration in Normal and Diabetic Rat Testes. (Rat-Ova Test).

Testes	Normal		Diabetic		Significance of Diff. of means
	No. of rats	Time to denude ova (min)	No. of rats	Time to denude ova (min)	
Chilled, homogenate	5	46 \pm 8.2*	5	66 \pm 15.2	P = .30
Incubated, homogenate	5	30 \pm 14.6	5	24 \pm 12.7	P = .75
+ Sem. ves. incubated	5	35 \pm 9.3	5	46 \pm 10.8	P = .65

* Standard deviation.

Methods. Adult male rats were made diabetic by injecting alloxan intraperitoneally (175 mg/kg body weight). Qualitative determinations for glycosuria in the alloxan treated rats were made. In some cases quantitative blood sugar determinations³ were performed because it was thought that hyaluronidase concentration might vary with the degree of hyperglycemia. Body weights were obtained before and after alloxan administration; after 30 days the testes of each rat were removed and assayed for hyaluronidase.

One of the two assay procedures used was the turbidimetric method as previously described for chilled testis homogenates.⁴ Since it has been reported that incubation of the testis homogenate increases the yield of the enzyme,⁵ a series of experiments was performed with the additional step of incubation. The amount of enzyme is reported as "Turbidity reducing units" (T.R.U.). The other assay procedure, the rat-ova test, was modified

from the original procedure⁶ in that comparisons were made of the time required for denudation of rat ova of their follicle cells. Saline extracts of testes from normal and diabetic rats were prepared in concentrations which were the same for both the experimental and control testes in each assay.

An additional test was included in this study. Comparisons were made in the yields of enzyme produced in the experimental and control groups when macerated seminal vesicles were mixed with the testis homogenates before extracting. It was shown that increased yields of enzyme could be obtained when the seminal vesicles were added.⁷

Results. The hyaluronidase concentration of the testes of normal and diabetic rats are presented in Tables I and II. In no case was the enzyme concentration in the testes of the experimental and control rats significantly different. When the testis homogenate was incubated before extraction an increase in enzyme concentration was obtained in both groups. In the rat ova test, the amount of extract in gram-equivalents of testis material was purposely lowered when the testis homogenate was incubated, otherwise the reaction would have proceeded so rapidly that an ac-

³ Sumner, J. B., *J. Biol. Chem.*, 1925, 65, 393.

⁴ Leonard, S. L., Perlman, P. L., and Kurzrok, R., *Endocrinology*, 1946, 30, 261.

⁵ Perlman, P. L., Leonard, S. L., and Kurzrok, R., *Endocrinology*, 1948, 42, 26.

⁶ Leonard, S. L., and Kurzrok, R., *Endocrinology*, 1946, 30, 85.

⁷ Leonard, S. L., Perlman, P. L., and Kurzrok, R., *Endocrinology*, 1947, 40, 199.

curate comparison of the time of denudation of the ova would be impossible. With the chilled testis homogenates equivalents of .05 g of tissue were employed and with the incubated testes .01 g of tissue.

In the experiments where the seminal vesicle material augmented the yield of hyaluronidase no significant difference was observed between the amount of enzyme obtained from normal and diabetic animals (Tables I and II). The amount of enzyme present in the seminal vesicle was practically negligible. In the rat-ova test .005 g of tissue equivalent were required to denude the ova in the time indicated.

Although the diabetic rats exhibited hyperglycemia during the period of the experiments no correlation was observed between blood sugar levels and the enzyme concentrations of the testes. No histological changes were observed in the testes of the experimental rats, and there was no altered breeding per-

formance as measured by the size of the litters sired.

In contrast to these results destruction of the germinal epithelium by hypophysectomy or cryptorchidism is followed by a marked decrease in hyaluronidase levels.⁸ Whether the enzyme level of the germinal cells of the testes can be altered by physiological changes which do not destroy these germ cells remains to be shown.

Summary. 1. Alloxan diabetes in the male rat had no effect on the hyaluronidase concentration of the testes.

2. Augmentation of the yield of extractable hyaluronidase from testis homogenates by seminal vesicle homogenates was confirmed. No difference in augmentation was noted between the normal and diabetic rat testes.

⁸ Leonard, S. L., Perlman, P. L., and Kurzrok, R., *Endocrinology*, 1948, 42, 176.

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⁵ Perlman, P. L., Leonard, S. L., and Kurzrok, R., *Endocrinology*, 1948, **42**, 26.

⁶ Leonard, S. L., and Kurzrok, R., *Endocrinology*, 1946, **39**, 85.

⁷ Leonard, S. L., Perlman, P. L., and Kurzrok, R., *Endocrinology*, 1947, **40**, 199.

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Relaxin in the Ovary of the Domestic Sow (*Sus scrofa* L.).*

F. L. HISAW AND M. X. ZARROW.

From the Biological Laboratories, Harvard University, Cambridge, Mass.

The recent work of Albert, Money and Zarrow¹ indicates that a much higher concentration of relaxin is found in the whole, fresh ovary of the sow than in the defatted, dried corpora lutea.²⁻⁴ They¹ also found that the hormone is distributed throughout the ovary with the exception of the follicular fluid, and that during pregnancy its concentration is 500 to 1500 times greater than during a

normal estrous cycle. The present investigation was undertaken for the purpose of determining quantitatively the relaxin content of the ovary throughout pregnancy and also to find when relaxin first appears in the ovary both during pregnancy and the normal estrous cycle. In addition, studies were made of the blood, uterus and placenta for relaxin content.

*Aided by a grant from the United States Public Health Service to Professor Frederick L. Hisaw.

¹ Albert, A., Money, W. L., and Zarrow, M. X., *Endocrinol.*, 1947, **40**, 370.

² Fevold, H. L., Hisaw, F. L., and Meyer, R. K., *J. Am. Chem. Soc.*, 1930, **52**, 3340.

³ Abramowitz, A. A., Money, W. L., Zarrow, M. X., Talmage, R. V. N., Kleinkholz, L. H., and Hisaw, F. L., *Endocrinol.*, 1944, **34**, 103.

⁴ Albert, A., Money, W. L., and Zarrow, M. X., *Endocrinol.*, 1946, **30**, 270.

Experimental procedure and methods. In order to avoid the possible loss of relaxin as a consequence of the number of manipulations in the extraction of the tissues, purification was carried out only to the point of furnishing a preparation suitable for assay purposes. Essentially the technic used was that described as step No. 1 by Albert, Money and Zarrow.¹ The tissues were obtained on the slaughtering floor and at the same time data were secured as to the age of the sow, condition of the ovary (follicles or corpora

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TABLE I.
Concentration of Relaxin in Blood, Placenta, Ovary, and Other Tissues of the Sow.

Age of pig	Tissue	Stage of cycle or length of fetus	No. of tests	Relaxin content G.P.U.
Gilt	Ovary	Immature	4	<1.0 per g
Mature	"	Follicular	2	<1.0 " "
"	"	Luteal	3	2.5 to 5 " "
"	Follicular fluid	Follicular	2	<1.0 " 2 ml
"	Placenta	4½-inch fetus	1	0.5 " g
"	"	6 " "	2	2.5 " "
"	"	10 " "	2	0.5 " "
"	Blood	5½ " "	1	2.0 " 1 ml
"	"	7 " "	2	2.0 " " "
"	Uterus	Pregnant	1	<1.0 " g
"	Thymus	—	1	<0.1 " "
"	Thyroid	—	1	<0.1 " "
"	Liver	—	1	<1.0 " "
"	"	—	1	<0.1 " "

viously is applicable to the present material.

Assays of the ovaries of pregnant sows (Fig. 2) indicate that the relaxin concentration rapidly increases during early pregnancy, and reaches a plateau at approximately 10,000 G.P.U. per gram of fresh ovary when the fetus is about 5 inches in length. The earliest stage of pregnancy studied was one in which the fetuses were $\frac{3}{8}$ of an inch in length. The ovaries of this sow contained 500 G.P.U. of relaxin per gram of fresh tissue. Therefore, the relaxin content of the ovary increased some 20-fold between this early stage and that at which the fetuses attained a length of 5 inches.

An extract of the ovaries of a sow with fetuses 9½ inches long was used for making a comparison of the guinea pig and mouse assay procedures. The guinea pig assay was carried out as previously described. In the mouse assay, groups of 15 castrated female mice previously primed with estradiol were used and a unit defined as that amount of relaxin which would produce relaxation in the symphyses of two-thirds of the animals. There was complete agreement between the results of the two methods of assay as both showed a concentration of 10,000 G.P.U. of relaxin per gram of fresh ovary.

In view of the extremely high concentrations of relaxin in the ovaries of the pregnant sow it was felt desirable, for the purpose of comparison, to examine the ovaries of the gilt

and of the sow during a normal estrous cycle. The tissues were collected and assayed by the guinea pig method in the same manner as described above. These assays gave negative results, at a level corresponding to 1 G.P.U. of relaxin per gram of fresh tissue, for both the ovary of the gilt and that of the sow in the follicular phase of the estrous cycle. Examination of the follicular fluid also gave negative results. However, during the luteal phase, the ovary was found to contain 2.5 to 5 G.P.U. per gram.

The relaxin content of the blood, placenta and other tissues of the pregnant sow also was determined (Table I). Several assays were carried out on the blood serum of 2 pregnant sows, one of which had fetuses of 5½ inches and the other fetuses of 7 inches. In both cases the blood was found to contain 2 G.P.U. per ml. The placentas of 3 animals having fetuses 4½, 6, and 10 inches in length were found to contain 0.5 to 2.5 G.P.U. per gram and no correlation with length of gestation was observed. Assays for relaxin in the uterus, thyroid, thymus and liver gave negative results.

Discussion. These observations indicate that relaxin is primarily a hormone of pregnancy. It is found in the ovary in small amounts (2.5 to 5.0 G.P.U. per g) during the estrous cycle, associated with the presence of a functional corpus luteum, but it begins to increase very early in pregnancy

lutea) and, if pregnant, the length of the fetus. The tissues were brought immediately to the laboratory and the preparation of the extracts for assay was started.

The tissues were first ground and extracted in the cold with 6 volumes of 3% HCl for 48 hours. At the end of the first extraction period the mixture was made up to 4% NaCl and the supernatant fluid separated from the residue by centrifugation. The residue was then re-extracted in the same manner for an additional 24 hours and discarded. The two supernatant fluids were combined, adjusted to pH 7.0 and dialyzed against running tap water. Adequate dilutions were prepared and the sample assayed in castrated guinea pigs by means of the relaxation of the symphysis pubis. In a few instances when the extract was too dilute, the preparation was concentrated by adding 5 volumes of cold acetone and dissolving the precipitate in a suitable volume of water.

In the present assay procedure the guinea pigs were injected with 1 μ g of estradiol daily for 3 days instead of the usual 4 days and the material to be tested was injected on the morning of the fourth day. Six hours later the pelvis of the animals were palpated according to the technic of Abramowitz, *et al.*⁴ A guinea pig unit (G.P.U.) was defined as that amount of relaxin which produced relaxation of the symphysis pubis in two-thirds of a group of 12 or more guinea pigs. The assay was carried out by preparing a series of dilutions and determining the concentration of relaxin which gave a unit response.

It is important to note that several precautions were carefully observed. Only animals in good nutritional state and weighing 400 to 800 g were used. Furthermore, all guinea pigs were palpated prior to the start of the estradiol treatment and also just prior to the injection of the sample of relaxin. In both instances all guinea pigs with relaxed or questionably relaxed pelvis were discarded. Finally, it is important to mention that it is possible to distinguish gradations in the response of the guinea pigs. However such distinctions may lead to considerable error

as the test is in a large measure based on subjective judgment. Consequently, only an unquestionable relaxation of the symphysis was accepted as a positive response while doubtful or non-relaxed symphyses were considered negative.

Results. Ovaries were obtained from 15 pregnant sows with fetuses ranging in length from $\frac{3}{8}$ to $10\frac{3}{8}$ inches and assayed for relaxin content on a total of 510 castrated guinea pigs. Prior to carrying out these assays, an extract was prepared of a single ovary from a sow in early pregnancy and a dose-response curve obtained. It may be seen in Fig. 1 that the results give a typical sigmoid curve similar in shape to the curve obtained with extracts of dried corpora lutea,³ thus indicating that the assay reported pre-

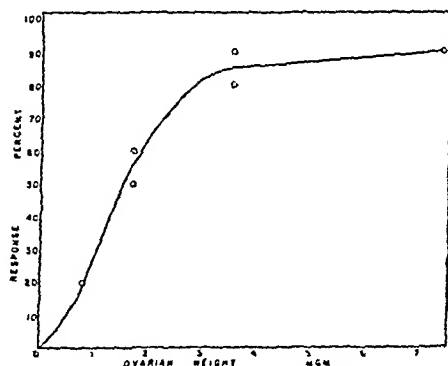


FIG. 1.

The dose-response curve of relaxin obtained from the ovary of a pregnant sow. Mg equivalent of fresh ovary are plotted against the percentage of guinea pigs showing pubic relaxation.

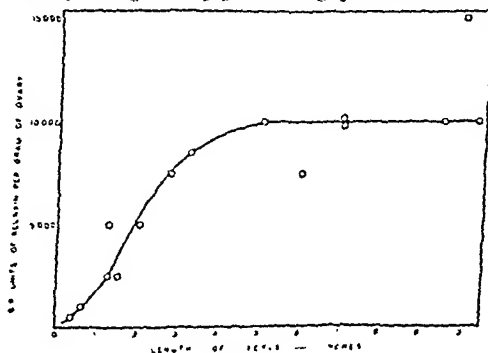


FIG. 2.

The relaxin content of the ovary of the pregnant sow plotted against the average length of the fetuses as an indication of the stage of pregnancy.

TABLE I.
Selection of Patients with Pneumococcal Infections.

Age, yrs	Pre-existing complications	Days of illness on which observations were made	Additional protein therapy	Recovered
Primary Meningitis.				
32	None	1,3	Serum	Yes
38	Pituitary adenoma	1,4	"	"
50	Skull fracture	1,6	"	"
60	Cirrhosis	4,7	"	No
Primary Pneumonia				
46	None	18,25,79		Yes
28	"	4,6,14,33	Gamma globulin	"
34	"	2,4	" "	"
39	Alcoholism	21,36	Serum	"
57	"	21,24,49	"	"
46	" chr. osteomyelitis	4		No
49	"	19		Yes
70	"	10,14,31,46	Gamma globulin	"
62	" bronchiectasis	11,15,29	" "	"
42	"	8,9	" "	"
43	"	7,8,17	" "	"
79	Congestive failure	8,17,24		No
70	Emphysema	14,28		Yes
71	Heart disease	3,21		"
74	Congestive failure	5,6,32	" "	"
78	Heart disease	36		"
69	Diverticulitis	6		"
74	Cachexia senilis	4		"
65	Multiple myeloma	1,5 (months)		"

tures and burns.³ Leutscher⁴ reported greatly decreased electrophoretic albumin and increased fibrinogen concentrations in lobar pneumonia, as well as lesser increments in beta and gamma globulins. Pleural fluids similarly fractionated were found to contain the same constituents as serum, but in lower concentrations.

Selection of patients. During 1946, 23 patients with pneumococcal infections were selected from the Medical Clinic of the Rochester Municipal and Strong Memorial Hospitals for this study. As indicated in Table I, 4 of these patients had primary meningitis, and 19 pneumonia. In addition to routine treatment with sulfadiazine and/or penicillin, 6 were also treated with type specific rabbit antiserum because of the severity of the infection, and 7 others were given supplemental intramuscular injections of immune human gamma globulin* to investigate its effects.

Methods. The venous blood samples were drawn in dry oxalate flasks under fasting conditions to minimize lipemia, and insofar as possible with only momentary stasis. All but 3 plasma samples had a specific gravity of 1.022 to 1.026 (copper sulphate method); one from a patient with pneumonia and hepatic cirrhosis was too low to read on one day and 1.021 when repeated the following day, and 2 others were 1.029 from acutely ill dehydrated patients. Electrophoresis was carried out in the tall form of the 11 ml Tiselius cell, in diethyl-barbituric acid buffer of pH 8.5 and ionic strength of 0.1. Plasma was diluted with an equal volume of buffer and dialyzed through cellophane against 2 liters of similar buffer. Current was passed for 3 hours with a field strength of 6.3 volts per

* The immune human gamma globulin was furnished by Sharpe and Dohme, Philadelphia, through the kindness of Dr. Charles A. Janeway, Boston. 50 ml of this material provided the antibody equivalent of 1200 ml of pooled normal human plasma, or not quite one-half of the estimated circulating gamma globulin in the blood.

³ Stern, K., and Reiner, M., *Fate J. Biol. and Med.*, 1946, 10, 67.

⁴ Leutscher, J. A., *J. Clin. Invest.*, 1941, 20, 99.

and attains a maximal concentration of approximately 10,000 G.P.U. per g of ovarian tissue by the time the fetuses are 5 or 6 inches in length. It is rather surprising, in view of this high concentration in the ovaries, to find only 2 G.P.U. of the hormone per ml of blood serum in the pregnant animal. This is only one-fifth of the concentration found in the blood serum of pregnant rabbits.⁵ However, it is 4 times the concentration found in pregnant guinea pigs⁶ which indicates a wide species variation.

While the ovary must be regarded as the major source of relaxin in the pregnant sow, small amounts (0.5 to 2.5 G.P.U. per g) were also found in the placenta. Compared to the concentrations obtained in the ovary this amount is extremely small; nevertheless it is significant to note that in both the guinea pig⁶ and the rabbit,⁷ relaxin has been found in the placenta.

⁵ Marder, S. N., and Money, W. L., *Endocrinol.*, 1944, **34**, 115.

⁶ Zarrow, M. X., *PROC. SOC. EXP. BIOL. AND MED.*, 1947, **66**, 488.

⁷ Zarrow, M. X., Unpublished observations.

The lack of relaxin in the ovary of the gilt and in the follicular fluid and ovary of the sow in pre-oestrus indicates that the corpora lutea are of importance in the formation of relaxative substance. The results of Albert, Money and Zarrow¹ show that relaxin is present in both the corpora lutea and the non-luteal tissue of the ovary of a pregnant sow. This may indicate that relaxin is formed in the luteal tissues and diffuses out to the surrounding area or that there is an extra-luteal site which forms relaxin under the stimulation of a corpus luteum.

Summary. No relaxin is found in the ovaries of the gilt nor of the sow during the follicular phase of the estrous cycle. There is, however, 2.5 to 5 G.P.U. of relaxin per gram of ovarian tissue present during the luteal phase of the cycle. The amount of relaxin in the ovaries increases rapidly during pregnancy and reaches a maximal concentration of approximately 10,000 G.P.U. per gram of tissue by the time the fetuses attain a length of 5 or 6 inches. The blood at mid-pregnancy contains 2 G.P.U. per ml while the placenta has 0.5 to 2.5 G.P.U. per gram.

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Electrophoretic Changes in Plasma Proteins in Patients with Pneumococcic Infections.

ROBERT A. BRUCE AND ERIC L. ALLING. (Introduced by W. S. McCann.)
(with the technical assistance of Anne D. Barnett.)

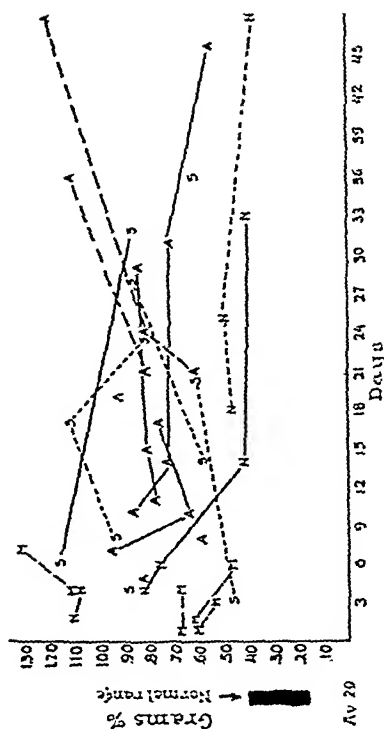
From the Chest Laboratory of the Department of Medicine and the Departments of Radiology and Medicine, University of Rochester School of Medicine and Dentistry, and the Medical Clinic of the Strong Memorial and Rochester Municipal Hospitals, Rochester, N. Y.

Alterations of the plasma proteins in relation to successive stages of pneumococcic infections (either pneumonia or meningitis), and to important antecedent factors influencing protein metabolism, are reported in this paper. Blix,¹ who studied the plasma of pneumonia patients electrophoretically, found that the alpha-globulin concentration was more than

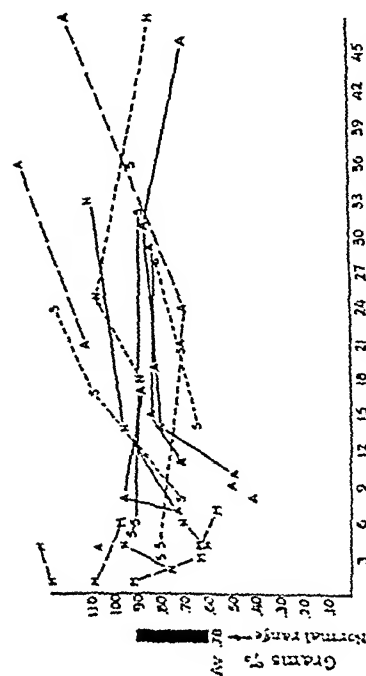
twice normal, and gamma globulin was reduced from 76 to 64% of the total globulin concentration. Longworth² confirmed these findings concerning alpha-globulin, and subsequent observers have shown that alpha-globulin generally increases in febrile reactions associated with tissue destruction, such as infectious diseases, coronary thrombosis, frac-

¹ Blix, G., *Z. f. die gesamte Exp. Med.*, 1939, **105**, 595.

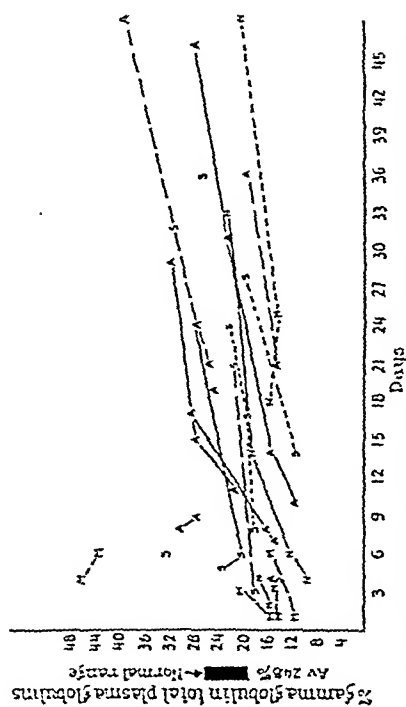
² Longworth, L. G., Shedlovsky, T., and MacInnes, D. A., *J. Exp. Med.*, 1939, **70**, 399.



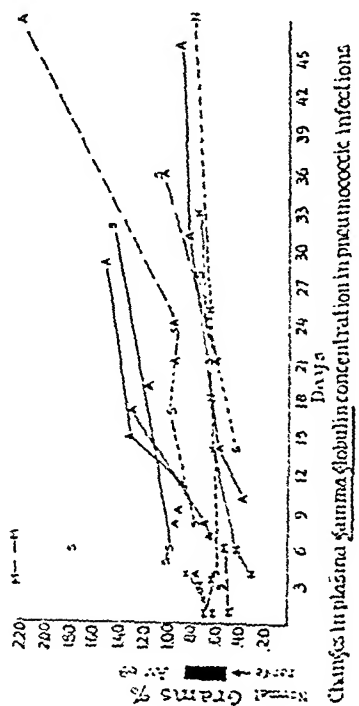
Changes in plasma fibrinogen concentration in pneumococcal infections



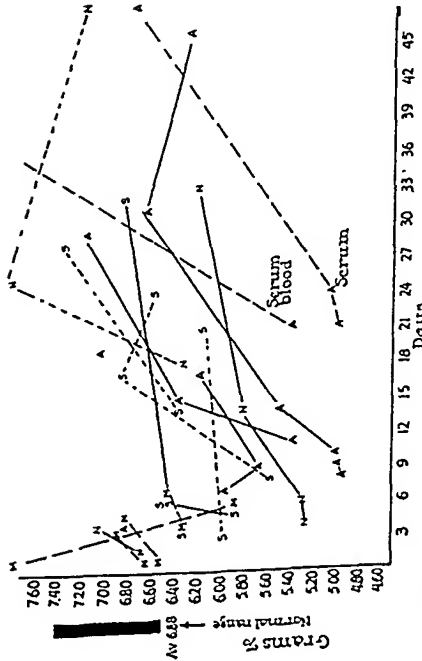
Changes in plasma beta globulin concentration in pneumococcal infections



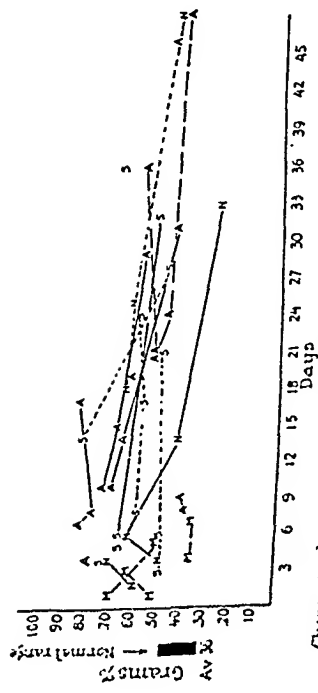
Changes in gamma globulin concentration in combined globulin fractions in pneumococcal infections



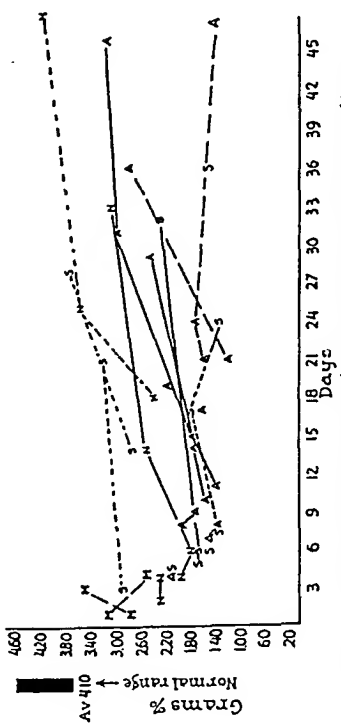
Changes in plasma gamma globulin concentration in pneumococcal infections



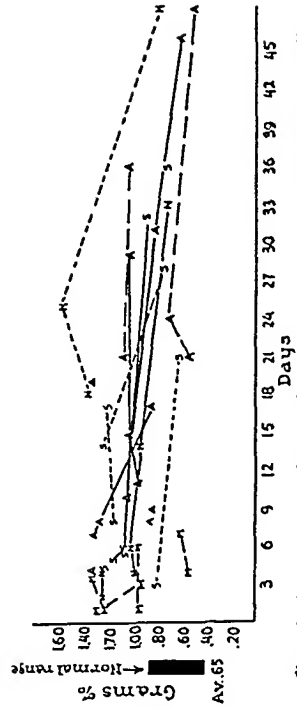
Changes in plasma total protein concentration in pneumococcal infections



Changes in plasma alpha 1 globulin concentration in pneumococcal infections



Changes in plasma albumin concentration in pneumococcal infections



Changes in plasma alpha 2 globulin concentration in pneumococcal infections

Fig. 1. Serial Changes in Plasma Protein Fractions in Pneumococcal Infections.

hepatic cirrhosis; 2 had heart failure with hepatic congestion; and 1 had multiple myelomata. Thus the abnormal increments in the gamma globulin levels appeared to be related to underlying disease of the liver or bone marrow. In the electrophoretic fractionation of the plasma proteins in the myeloma blood, there was a protein having the mobility of gamma globulin with a concentration of 5.9 g% as determined by the area under the peak. When this patient's plasma was studied again 5 months later, there was no change in this peak. Only 2 patients had subnormal gamma globulin levels. Each of these gave a history of previous pneumonic infections several years before. Both were given 50 ml of immune human gamma globulin intramuscularly in addition to the routine treatment. Subsequent electrophoretic patterns of their blood showed increments in the gamma globulin fraction. Four out of 5 other patients similarly treated with intramuscular gamma globulin showed appreciable increases in the plasma concentrations of this fraction following the injection (Fig. 1). The amount of gamma globulin injected in these 7 patients represented about 7.0 g, and the maximum average increase in concentration was 0.45 g%, or about 65% of the average normal level. Absorption was delayed as there was no prompt initial rise. Since, however, 4 of the 7 so treated were known to have had chronic alcoholism and another had had decompensated heart disease, it is difficult to attribute the greater increments in this fraction to the material administered, although it is possible that the restoration of normal levels was significant in the only 2 patients with abnormally low initial gamma globulin levels. During convalescence there was a relative increase in the gamma globulin concentration in all the patients studied, but this was attributed in part to the concomitant decline in alpha-globulins.

Discussion. Pneumococcic infections, in common with other acute infectious diseases and severe bodily trauma,³ initiate non-specific alterations in the protein metabolism. The present findings of hypoalbuminemia, increased alpha-globulins and fibrinogen are in

accord with the findings of previous observers.^{1,2} When evaluated chronologically, however, from the onset of the infection, it is apparent that the alpha-globulins and fibrinogen increase promptly and decline gradually in convalescence; whereas the hypoalbuminemia becomes progressively more severe, reaching the lowest levels in the second week of the infection and slowly is restored to normal over the following month. The intensity of the hypoalbuminemia, and in turn the hypoproteinemia, is considerably influenced by antecedent factors adversely affecting liver function, such as malnutrition, chronic alcoholism, cirrhosis and chronic passive congestion. Furthermore the severity of the electrophoretic hypoalbuminemia is greater than is recognized by chemical determinations. Dole⁷ has shown that the Howe separation⁸ is faulty, due largely to the solubility of much of the alpha-globulins in the concentration of sodium sulfate employed in the Howe technic. Hence the hypoalbuminemia tends to be obscured by the concomitant increase in alpha-globulins during acute febrile illnesses which are incompletely salted out by the clinical method of separation. The hypoalbuminemia in pneumonia may be the result of poor dietary intake of nitrogen, nitrogen catabolism, losses of protein in exudate and urine, and possibly impaired synthesis of protein. In experimental dogs, poor dietary protein intake and plasmapheresis have been shown to diminish the albumin level and increase the alpha-globulins.⁵

Previous variations in the reported findings of gamma globulin concentrations in pneumonia^{1,4} may have been due to the selection of patients and the relation to the onset of infection. Our findings indicate abnormally low levels early in the infection in occasional patients, a relative increase in concentration during convalescence, and an abnormally high level in those patients having impaired liver function prior to the onset of pneumococcic infection. The supplemental intramuscular

⁷ Dole, V. P., and Braun, E., *J. Clin. Invest.*, 1944, **23**, 708.

⁸ Howe, R. E., *J. Biol. Chem.*, 1921, **49**, 109.

cm. Further details of this technic may be found in a previous paper.⁵

Results. The alterations in plasma proteins determined electrophoretically in relation to the duration of the pneumococcal infection are shown in Fig 1. In addition, the symbols indicate patient-groups: primary meningitis (M), senile (S), history of chronic alcoholism (A), and those whose previous health was normal (N). Differences in therapy are indicated by the connecting lines: small broken lines indicate routine treatment; large broken lines indicate supplemental use of anti-serum; and solid lines indicate those treated with gamma globulin. For comparative purposes the range and mean values for the same fractions in 17 normal individuals are indicated in each graph.

All *total protein* levels obtained initially were somewhat depressed, except for 1 patient with meningitis and dehydration (plasma specific gravity 1.029). The blood samples from the meningitis group were obtained early in the infection and exhibited an average level of 7.07 grams%, whereas those with pneumonia were obtained later in the first and second weeks of the illness and had an average level of 5.88 grams%. There was considerable scatter, with a tendency to reach the lowest levels in the second week, and then to increase during convalescence. This parallels the observations of Rutstein, *et al.*, on plasma volume alterations in pneumonia.⁶ The pneumonia patients with an alcoholic background had an average total protein concentration of 5.55 g%, whereas the non-alcoholic group averaged 6.07 g%. These values are based on samples taken from 2 to 36 days in the course of the illness, and hence are not strictly comparable. The lowest total protein of 4.9 g% was obtained from an alcoholic patient however.

The plasma *albumin* levels were low in all the initial blood samples studied. Hypoalbuminemia progressed until the second week of

the infection, and all but 2 patients continued to exhibit a subnormal albumin concentration up to a month after the onset of the infection. The lowest level for non-alcoholic patients was 1.76 g%, whereas the elderly and alcoholic groups exhibited a more marked hypoalbuminemia ranging from 1.35 to 1.74 g% during the first 2 weeks. This is in contrast to the normal range of 3.7 to 4.6 g% by this method.

The *alpha-globulins* were increased up to twice normal concentrations during the first week. The 2 exceptions to this trend were patients with advanced cirrhosis, one of whom died of hepatic insufficiency. The increased alpha-globulin levels gradually declined to normal in over a month. The *beta globulin* levels varied considerably; the highest initial level occurred in a patient with pituitary adenoma who had an intracranial-paranasal fistula complicated by meningitis; and the lowest levels were in 2 patients with chronic alcoholism. During convalescence the beta globulin showed marked increases in 3 patients, one of whom was an elderly man with a history of congestive heart failure who suffered 3 relapses of pneumonia before a fatal outcome; and the other 2 were chronic alcoholics with pneumonia treated with antipneumococcal rabbit serum.

The "*fibrinogen fraction*" was considerably increased in all patients, and returned to normal during convalescence in only 2 patients. These 2 had pneumonia without any antecedent complications. The highest value of 1.3 g% was obtained just prior to death in the patient with meningitis and hepatic insufficiency. Two others, who showed progressive hyperfibrinogenemia, had chronic alcoholism, were severely ill with pneumonia, and showed jaundice. It should be noted that the fibrinogen values obtained by electrophoresis contained unknown amounts of gamma-1 globulin, so that the increases observed cannot be considered pure fibrinogen. Hence we have used the term "fibrinogen fraction."

Gamma globulin concentrations of greater than 0.9 g% were seen in the acute phase of the illness or in convalescence in 10 patients. Of these, 4 had chronic alcoholism; 3 had

⁵ Zeldis, L. J., and Alling, E. L., *J. Exp. Med.*, 1945, **81**, 515.

⁶ Rutstein, D. D., Thomson, K. J., Tolmuck, D. M., Walker, W. H., and Floody, R. J., *J. Clin. Invest.*, 1945, **24**, 11.

TABLE I.
Results of Albumin Administration in Patients with Pneumococic Lobar Pneumonia.
A. Effects on Plasma Albumin Levels and Protein Metabolism.

Patient	Day of illness when alb. was started	Plasma albumin conc., g/100 ml			Protein intake, g		Apparent retention in protein during period of observation, g
		Before	After	1 wk later	Dietary	Alb. i.v.	
J.S.	Control	2.65	2.85	2.88	542	—	62
C.M.	"	3.20		3.24	*	—	—
D.P.	5	2.48	3.46	3.48	611	100	245
W.D.	10	2.68	3.74	3.91	743	100	—
A.M.	7	2.51	3.64	3.78	743	100	244
S.W.	11	1.58	3.19	3.23	472	125	151
E.E.	9	1.25	2.26	—	890	125	598
C.S.	7	2.54	4.01	3.32	986	170	363

*This patient was permitted an unrestricted diet, hence the nitrogen intake could not be determined.

B. Effects on Plasma Globulin Levels.

Patient	Alpha-1		Alpha-2		Beta		Fibrinogen		Gamma	
	b*	a	b	a	b	a	b	a	b	a
J.S.	.66	.52	1.33	1.19	.79	.95	.95	.89	.58	.75
C.M.	.58	.58	1.10	1.24	.87	.92	.97	.88	.60	.96
D.P.	.69	.51	1.29	.92	.92	.75	.72	.45	.80	.58
W.D.	.67	.46	1.21	.91	1.02	.95	.67	.58	.99	.74
A.M.	.64	.47	.98	.76	.73	.82	.71	.50	.60	.82
S.W.	.60	.47	.99	.86	.91	.93	.82	.92	1.01	1.14
E.E.	.65	.58	.95	.88	.82	.71	.93	1.23	1.06	1.18
C.S.	.48	.35	.95	.70	1.09	.87	.93	.50	.70	.65

*"b" represents the concentration before the albumin was administered, and "a" represents the concentration after the albumin.

cretion, whereas the fecal nitrogen excretion was estimated to be 1.0 g per diem. Such a simplified nitrogen balance study was maintained for 5 to 7 days during which time the treated patients received 25 g (100 ml) of salt-poor human albumin[‡] intravenously daily for 4 to 6 days. Venous blood samples for plasma electrophoresis were obtained the morning before, the morning after, and again a week after the course of albumin. The technique of electrophoresis was the same as previously described.^{1,2} Electrophoresis of the plasma was done on the second control patient who was permitted an unrestricted hospital diet *ad libitum*. Serial determinations of pulse rate, blood pressure, venous pressure, respiratory rate, breath-holding time,

cardiac output (ballistocardiographic method[§]), and pulmonary capacities including total and vital capacity as well as residual air were made before and after the administration of albumin intravenously in 5 patients.

Metabolic results. Table I summarizes the changes in concentration of the various plasma protein fractions and the alteration of nitrogen balance resulting from the infusion of albumin in 6 patients with pneumonia. The treated patients exhibited increments in albumin concentration of 0.98 to 1.61 g% following the administration of 100 to 170 g of albumin over 4 to 6 days, and in most instances these increments were maintained over the following week of observation. In contrast the controls showed but slight change in albumin levels. Furthermore, the treated patients retained more protein than could be accounted for by the amount of albumin

‡ The salt-poor human albumin was furnished through the kindness of Mr. R. B. Clark, by the Cutter Laboratories, Berkeley, Calif.

‡ Zeldis, L. J., Alling, E. L., McCoord, A. B., and Kulka, J. P., *J. Exp. Med.*, 1945, **82**, 157.

§ Courtesy of Dr. H. R. Brown, Jr., of Department of Medicine.

injections of gamma globulin† may have been responsible for the increases in plasma levels which occurred in the 2 patients with low values.

Summary. Multiple electrophoretic determinations of the plasma proteins of 23 patients with pneumococcic pneumonia or meningitis have been made in relation to the

† Dr. W. Addison Clay assayed the protective power of gamma globulin in type 27 pneumococcic peritonitis in 40 mice and found a reduction of gross mortality from 95 to 55%. By serial dilutions the protective power was estimated to be of the order of one ten thousandth of that of immune rabbit serum.

duration of the infection and the importance of antecedent factors. Hypoproteinemia, due largely to significant progressive hypoalbuminemia, together with early increases in alpha-globulins and fibrinogen have been observed. Malnutrition, chronic alcoholism, cirrhosis and chronic passive congestion of the liver were found associated with more severe degrees of hypoalbuminemia. Diminished plasma albumin levels were seldom restored to normal within a month from the onset of the infection. The effects of intramuscular injections of immune human gamma globulin were investigated, with inconclusive results.

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Effects of Albumin on Hypoalbuminemia in Pneumococcic Pneumonia.

ROBERT A. BRUCE* AND ERIC L. ALLING. (Introduced by W. S. McCann.)
(with the technical assistance of Anne D. Barnett.)

From the Chest Laboratory of the Department of Medicine, and the Department of Radiology and Medicine, University of Rochester School of Medicine and Dentistry, and the Medical Clinic of the Strong Memorial and Rochester Municipal Hospitals, Rochester, N.Y.

A previous report¹ confirmed the findings of others that patients with pneumococcic infection exhibit hypoalbuminemia within the first 2 weeks of the infection. Results obtained by electrophoresis of plasma proteins showed depression of albumin concentrations to as low as 1.4 to 1.8 g%, and that normal concentration was not often established within a month after the infection. Such marked hypoalbuminemia was considered to be the result of protein loss in exudate and urine in addition to protein break-down and malnutrition during the acute phase of the illness. Because of this hypoalbuminemia the effects of intravenous albumin therapy were investigated. This report presents the metabolic changes as well as some of the secondary

effects of such therapy on cardio-respiratory functions.

Patients and procedures. During 1947 eight patients with severe pneumococcic lobar pneumonia were selected, and in addition to the routine chemotherapy of the infection 2 were controls, and 6 were treated with albumin. Observations on still another patient are included because of a fatal outcome following albumin therapy. The 6 albumin-treated patients and one control were placed on a special liquid diet† of known nitrogen content a day or two after the restoration of normal temperature following the febrile reaction. Twenty-four hour urinary collections were made to determine urinary nitrogen ex-

* Bertha Hochstetter Buswell Research Fellow in Medicine.

¹ Bruce, R. A., Alling, E. L., and Barnett, A. D., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 398.

† The diet consisted of 1200 ml of powdered whole milk, eggs, yeast, and sugar in addition to orange juice. There was 17.7 g of nitrogen in this mixture. To satisfy some patients, the amount of diet had to be increased to 2400 ml volume.

Histochemical Demonstration of Sites of Phosphamidase Activity.*

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In the course of experiments on the histochemical specificity of phosphatases the use of 18 out of 19 different substrates resulted in the production of identical patterns of enzyme distribution in all tissues and in all ranges of pH.¹ The nineteenth substrate, p-chloranilidophosphonic acid, included because of the existence of a specific "phosphamidase",²⁻⁴ yielded a pattern identical with the others at pH 9 and 7, but produced strikingly different pictures at pH 5. Since this finding is a strong evidence in favor of the existence of a specific enzyme, a technic was developed for its histochemical demonstration.

Experimental. p-Chloranilidophosphonic acid ($\text{p-ClC}_6\text{H}_4\text{NHPO}(\text{OH})_2$) was synthesized by the method of Otto.⁵ The crude product was dissolved in an excess of NH_4OH , filtered, and neutralized, under ice cooling, with acetic acid. A slight excess of acetic acid was added (to about pH 4), and the solution was placed in the refrigerator. In a few hours the free phosphonic acid precipitated almost quantitatively. It was filtered under suction, washed with ice cold water, and dried *in vacuo*. The dry powder was extracted with cold absolute alcohol to remove p-chloraniline and dried. A 0.1 M stock solution was prepared by dissolving a calculated amount

of the powder in an excess of 10% NH_4OH , adjusting the solution with dilute acetic acid to about pH 8 and filling it up to volume with distilled water. Such stock solutions were found to remain stable at refrigerator temperature for many weeks.

Thin slices of fresh tissue were fixed in a number of different fixatives, chilled acetone, 95% and absolute alcohol being found most satisfactory. Paraffin sections were incubated at 37°C from 2 to 48 hr in mixtures containing various amounts of substrate, $\text{Pb}(\text{NO}_3)_2$, and buffer in the range from pH 4.5 to 7.5. The best and most constant results were obtained at pH 5.4 to 5.8, the concentration of the substrate being 0.005 M, that of $\text{Pb}(\text{NO}_3)_2$, 0.0025 M and that of the buffer, 0.05 M. The presence of MnCl_2 in a concentration of 0.002 to 0.005 M greatly intensified the picture; Mg and Ca salts were without marked effect. Above pH 6.2 the reaction became distinctly weaker, disappearing altogether at pH 6.7. Above this pH level pictures corresponding to the distribution of nonspecific alkaline phosphatase were obtained. Below pH 5.3 confusing artifacts consisting in the Pb impregnation of various structures such as connective tissue and muscle fibers, nerves, mucin, etc. were often observed. These artifacts were present even in slides inactivated by dipping them for 5 minutes in Lugol's iodine solution prior to incubation, whereas true enzymatic reaction was completely abolished by this treatment. Since the substrate is not entirely stable around pH 5.6, the slides must be placed in the solution at an angle, the section facing downward, to avoid, as far as possible, the indiscriminate precipitation of Pb phosphate on the tissue. This maneuver will cause the precipitate to accumulate on the back surface of the slides from which it can be wiped off. Collodion coating of the slides interferes with

* This work has been done under grants from the Douglas Smith Foundation for Medical Research of The University of Chicago, and from the Pathology Study Section of the U. S. Public Health Service.

¹ Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1949, to be published.

² Walschmidt-Leitz, E., and Koehler, E., *Biochem. Z.*, 1933, **258**, 360.

³ Ichihara, M., *J. Biochem. (Japan)*, 1933, **18**, 87.

⁴ Bredereck, H., and Geyer, E., *Z. physiol. Chem.*, 1938, **254**, 223.

⁵ Otto, P., *Ber. deutsch. chem. Ges.*, 1895, **28**, 617.

alone. The magnitude of this protein retention is apparently dependent upon the dietary protein ingested, as well as the albumin administered. The patient (S.W.) who showed the least retention of protein, received only limited quantities of the special liquid diet for 2 days due to a misunderstanding of the orders. Contrariwise the patient (E.E.) who retained 598 g of protein during the period of observation was the one who requested and ingested more than the prescribed amount of the diet.

In addition to the changes in albumin concentration in the treated patients, there were reductions in the globulin fractions, especially the alpha-globulins (Table I). The mechanisms^{||} of these alterations in the various fractions of the plasma proteins are not clear from these studies. Suitable isotope studies yielding accurate data on rates of protein turn-over will be needed to determine whether there is impaired synthesis or utilization, of the albumin and globulins under such conditions of stress.

Cardio-respiratory results. One patient, clinically considered to have chronic pulmonary disease, basilar pneumonia and shock, was given 25 g of albumin intravenously. Within 3 hours this patient developed acute pulmonary edema. Electrocardiogram showed left bundle branch block and ischemic type of T waves. Before definitive therapy could be instituted the patient died. Autopsy showed coronary arteriosclerosis, cardiac dilatation and hypertrophy, myocardial scars and pul-

monary edema. Thus, the cause of the shock probably was due to coronary artery disease, and the administered albumin evidently contributed to pulmonary congestion. By electrophoresis the plasma albumin concentration was initially 2.0 g%. Because of this experience the secondary cardio-respiratory effects of albumin were investigated in all patients subsequently treated. Before any albumin therapy, each patient was found to have hypotension with systolic blood pressure ranging between 88 and 104 mm of mercury. Following the infusion of albumin, the effects were quite variable, even in the same patient on consecutive days. The trend of changes, however, were a slowing of the pulse together with increased blood pressure, stroke volume and cardiac output. The venous pressure often rose, but not above 12 cm of water. One patient (C. S.), who had had a myocardial infarction several months previously, showed no signs of cardiac dilatation or pulmonary congestion. The breath-holding time was often increased. Finally, the observed reductions in pulmonary volumes immediately after albumin therapy were indicative of pooling of blood within the lungs.

Summary. Six patients recovering from the acute febrile reaction of severe pneumococcal lobar pneumonia were given salt-poor human albumin intravenously. Electrophoretic analyses of the plasma proteins showed significant increases in the albumin concentration, together with reduction in globulins, particularly alpha-globulin, following this therapy. Simplified nitrogen balance studies showed retention of more protein than could be accounted for by the amount of albumin alone. Another patient with shock related to serious heart disease succumbed from pulmonary edema shortly after the administration of albumin. The secondary cardio-respiratory effects of albumin therapy were investigated in 5 patients.

^{||} There was no significant increase in plasma volume by the Evans blue technic in the patient who received 150 g of albumin (the authors are indebted to Dr. L. A. Kohn for this observation). By urinary chromatography there was no significant change in renal excretion of amino acids in another patient treated with albumin. (The authors are indebted to Dr. C. Dent of Department of Pathology for this observation.)

larities may be caused by improper fixation since they may show up in only one or two amidst a large number of perfectly well stained consecutive serial sections. Nonspecific impregnations may also occur; they can be recognized as such by incubating inactivated slides (treated for 10 minutes with Lugol's solution or with any N mineral acid) as controls. The inactivated sections will show the same artifacts.

In successful slides, however, there is a typical localization of the reaction, varying somewhat with different species. The enzyme is present mainly in the cytoplasm (often in a granular form) but also in some of the nuclei. Moderate amounts are found in many tissues such as the liver, where its distribution often but not invariably follows that of glycogen; in the secretory portions of the renal tubules; in the adrenal cortex; in the small intestine where its localization is similar to that of alkaline phosphatase; in the epithelial lining of the bronchi; in the beta cells of the pancreatic islets of the mouse (not constantly); in the lachrymal gland of the hamster; and many others. However, a very intense reaction, much stronger than at the sites mentioned, is obtained in two tissues: the grey matter of the central nervous system (especially that of the cerebellum), and in malignant tumors. Twenty-four adenocarcinomas of the gastrointestinal tract, 4 squamous carcinomas, 2 hypernephromas and a number of other epithelial tumors such as embryonic

carcinomas of the testis, cancers of the prostate, breast and bronchi, Walker rat carcinomas No. 256 and a butter yellow tumor stained very intensely and selectively. The reaction may start quite abruptly at the border of the malignant change, or the normal tissue may show a slight to moderate staining up to a distance of 1 or 2 mm from the edge of the neoplasm. In a few cases where the neoplasm showed various degrees of atypia in its different portions, the most atypical portions showed the most intense staining. So far not a single carcinoma has been found out of a total of 64 cases which did not show a sufficient reaction to set it off sharply against its environment. With sarcomas the results were much less constant. Some of them did not stain at all; others stained in an uneven, patchy way, and only a few stained as uniformly as carcinomas did. Two papillomas of the bladder, questionably malignant, stained moderately heavily. Granulomas such as sarcoidosis and human tuberculosis usually did not stain or showed a faint to moderate reaction in the giant cells and, rarely, even in the epithelioid cells. Tubercles in the guinea pig stained rather heavily.

Summary. A histochemical method for the demonstration of sites of phosphamidase activity is described. Small amounts of the enzyme are present in many normal tissues; large amounts are found in the grey matter of the central nervous system and in malignant epithelial tumors.

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Inhibition of the Shwartzman Phenomenon by Local Application of Bromobenzene and Other Solvents.*

LEWIS THOMAS AND CHANDLER STETSON, JR.

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When an intradermal injection of toxin derived from certain Gram-negative microorganisms is followed, after a period of ap-

proximately 20 hours, by an intravenous injection of similar bacterial toxin, a gross hemorrhage involving the entire injected skin area occurs. This hemorrhagic reaction has become known as the Shwartzman phenom-

* This work was aided by a grant from the Life Insurance Medical Research Fund.

the reaction; in fact, if the tissues happened to be embedded through collodion the latter must be removed before incubation by passing the slides through an alcohol-ether mixture or acetone.



FIG. 1.

Squamous carcinoma of the cervix uteri. Note abrupt start of reaction at the edge of the malignant change.



FIG. 2.

Adenocarcinoma of colon. Intense staining of carcinomatous glands; normal mucosa unstained.



FIG. 3.

Scirrhus carcinoma of breast. Note unstained ducts.

The procedure to be described is recommended.

1. Incubate slides at 37°C for 10 to 24 hr in the following mixture:

To 50 ml of 0.05 M maleate buffer pH \pm 5.6 (5.8 g of maleic acid + 62 ml of N NaOH in 1000 ml) add 1 to 1.5 ml of 0.1 M Pb (NO₃)₂ solution and a few drops of a 10% solution of MnCl₂; shake until the initial precipitate dissolves. Add 2 ml of 0.1 M phosphonate stock solution. Place mixture in a 45 to 60°C oven for about 30 minutes until the turbidity consisting of Pb₃(PO₄)₂ settles. Filter mixture into a Coplin jar. Support latter in an inclined position (at an angle of about 30°); place slides in it with the section facing downward.

2. Rinse slides in distilled water. Wipe precipitate from backs of the slides and around the tissue.

3. Remove superficial precipitate by placing slides in a 0.1 M citrate buffer of pH 4.5 to 5. As soon as the slide appears to be completely clear around the tissue, rinse it thoroughly under the tap. This differentiation is the most critical step in the procedure since insufficient treatment of the section may leave a precipitate, appearing black in the finished section, scattered all over the slide while overtreatment may remove part or all of the enzymatic reaction product.

4. Treat slides with ammonium sulfide, etc., as in the method for lipase.⁶

Results. The dependability of the method in its present form, as far as uniformity of results is concerned, is not entirely satisfactory. Usually, long series of sections from various tissues will stain quite uniformly. Occasionally, however, partial or total failures are observed. Some sections may fail to stain altogether; others will show a patchy distribution of the reaction; still others may show a reaction at an abnormal site such as the nuclei instead of the cytoplasm. It is a curious fact that the reaction will remain selective for the same cell or group of cells, regardless of differences in its intracellular localization. Some but not all of these irregu-

⁶ Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1945, 58, 362.

larities may be caused by improper fixation since they may show up in only one or two amidst a large number of perfectly well stained consecutive serial sections. Nonspecific impregnations may also occur; they can be recognized as such by incubating inactivated slides (treated for 10 minutes with Lugol's solution or with any N mineral acid) as controls. The inactivated sections will show the same artifacts.

In successful slides, however, there is a typical localization of the reaction, varying somewhat with different species. The enzyme is present mainly in the cytoplasm (often in a granular form) but also in some of the nuclei. Moderate amounts are found in many tissues such as the liver, where its distribution often but not invariably follows that of glycogen; in the secretory portions of the renal tubules; in the adrenal cortex; in the small intestine where its localization is similar to that of alkaline phosphatase; in the epithelial lining of the bronchi; in the beta cells of the pancreatic islets of the mouse (not constantly); in the lachrymal gland of the hamster; and many others. However, a very intense reaction, much stronger than at the sites mentioned, is obtained in two tissues: the grey matter of the central nervous system (especially that of the cerebellum), and in malignant tumors. Twenty-four adenocarcinomas of the gastrointestinal tract, 4 squamous carcinomas, 2 hypernephromas and a number of other epithelial tumors such as embryonic

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Summary. A histochemical method for the demonstration of sites of phosphamidase activity is described. Small amounts of the enzyme are present in many normal tissues; large amounts are found in the grey matter of the central nervous system and in malignant epithelial tumors.

16736

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From the Department of Pediatrics, Johns Hopkins University School of Medicine, and the Harriet Lane Home, Johns Hopkins Hospital, Baltimore, Md.

When an intradermal injection of toxin derived from certain Gram-negative microorganisms is followed, after a period of ap-

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proximately 20 hours, by an intravenous injection of similar bacterial toxin, a gross hemorrhage involving the entire injected skin area occurs. This hemorrhagic reaction has become known as the Schwartzman phenom-

enon, in acknowledgement of the investigator who originally described it.¹

During the course of recent studies on the mechanism of the Shwartzman phenomenon, it was found that the hemorrhagic reaction could be completely inhibited by the application of certain organic solvents to the prepared skin site, prior to the intravenous injection. It was also observed that these substances caused a marked increase in the permeability of the vessels of normal skin to circulating dye.

Materials and methods. Male white rabbits weighing from 1.8 to 2.5 kg, were used in all experiments. The entire abdomen was freed of hair by clipping and shaving at least 24 hours before the experimental day.

The bacterial toxins employed were 1) Meningococcal toxin No. 44B,†2) a purified polysaccharide derived from culture filtrates of *Serratia marcescens*,‡ and 3) an "agar washings" filtrate prepared from cultures of *B. coli* according to Shwartzman's technic.²

Each bacterial toxin was titrated in 3 or more rabbits to determine its skin preparing potency. Similar titrations were carried out to ascertain the optimal amount of each preparation for intravenous provocation of the phenomenon. The dose of each toxin to be used intradermally in the following experiments was arbitrarily chosen as that amount which, when injected intradermally in a volume of 0.5 cc, resulted in satisfactory skin preparation of at least 75% of the rabbits tested. The intravenous dose of each agent was chosen as that amount, which, when injected intravenously in a volume of 2.0 cc, elicited positive reactions in at least 75% of satisfactorily prepared rabbits.

In testing the inhibitory property of locally applied substances, two skin areas were prepared by the intradermal injection of toxin,

one on each side of the abdomen. These injections were spaced as far apart as possible, and the side used for testing inhibition was alternated in the rabbits of each experimental group in order to avoid the effect of possible differences in the natural susceptibility of the two sides of the abdomen.²

Results. The effect of a single application of bromobenzene was tested by applying this substance with a cotton swab over one side of the abdomen, at various times before and after an intradermal injection of bacterial toxin had been made on both sides. The intravenous injection of bacterial toxin was given 20 hours after the intradermal injection.

The inhibitory effect of bromobenzene is illustrated in Table I, in which it will be seen that when the local application was made at the same time as the intradermal injection of meningococcal toxin, or at various times thereafter up until the time of the intravenous injection, the Shwartzman phenomenon did not occur. Similar results were obtained in experiments using *S. marcescens* and *B. coli* toxins.

Maximum inhibition was brought about when bromobenzene was applied between 16 and 20 hours after skin preparation, as is indicated in Table I. When the skin was painted before the intradermal injection, no inhibition occurred; indeed, in a limited number of observations it appeared that the lesions in such areas tended to be more extensive than in unpainted control sites. When painting was delayed until 21 hours, or one hour after the intravenous injection, inhibition was not observed.

Various amounts of bromobenzene were dissolved in ether and tested 18 hours after skin preparation, in order to determine the concentration necessary for the effect. It was found that inhibition occurred regularly with 25 and 50% solutions, while the effect was less uniformly obtained with 5% and not at all with 1% solutions.

In subsequent experiments, other organic solvents were tested for similar inhibiting properties. Chlorobenzene, iodobenzene and benzene caused inhibition of the Shwartzman phenomenon, although the degree of inhibition was not as complete as with bromobenzene.

¹ Shwartzman, G., *Proc. Soc. Exp. Biol. and Med.*, 1928, 25, 560.

† Obtained from Dr. Gregory Shwartzman, and prepared by the method of this author.²

‡ Obtained from Dr. Murray Shear.

² Shwartzman, G., *Phenomenon of Local Tissue Reactivity*. Paul B. Hoeber, Inc., New York. 1937.

TABLE I.

Inhibition of the Shwartzman Phenomenon by Local Application of Bromobenzene at Various Intervals Before and After the Intradermal Injection of Meningococcal Toxin.

Bromobenzene applied to skin	No. of rabbits	Shwartzman reaction*		% inhibition
		Bromobenzene treated side	Untreated side	
24 hr before preparation	4	4*	4	0
4 " " " "	7	7	7	0
At same time as preparation	6	3	6	50
4 hr after " "	4	1	4	75
8 " " " "	4	1	4	75
16 " " " "	4	0	4	100
18 " " " "	6	0	6	100
20 " " " "	4	0	4	100
21+ " " " "	3	3	3	0

* Figures refer to number of rabbits showing hemorrhagic reaction at indicated skin area following intravenous injection of meningococcal toxin. Latter injection given at 20 hours after skin preparation.

† These rabbits received intravenous toxin one hour before the application of bromobenzene.

Chloroform and methyl salicylate, when applied 20 hours after the intradermal injection, caused inhibition in a majority of animals, but these agents yielded less constant results when applied at earlier intervals. Ether, acetone and alcohol had no effect on the Shwartzman phenomenon. In order to test the effect of a substance with primary skin irritative effects but without solvent action, 10% formalin was applied in a series of rabbits; this material had no effect on the phenomenon.

Observations were made on the local reaction of normal rabbit skin to single and repeated applications of bromobenzene. Within 30 to 60 minutes after a single painting, the skin exhibited a slight erythema. During the next few hours some edema occurred in the painted area in many of the animals. After 12 hours the skin usually appeared normal. However, after several days some superficial flaky desquamation of the skin commonly occurred. It was of incidental interest that the regrowth of hair on the shaven abdomen occurred more rapidly during the following weeks in areas which had been painted with bromobenzene. The effect of repeated painting with bromobenzene was similar to that following a single application, but the edema and desquamation were more extensive.

It is known that irritation of normal skin may cause an increase in local permeability to circulating dyes.³ The effect of bromoben-

zene on permeability was tested by applying this substance to the skin of normal rabbits which were simultaneously given an intravenous injection of 5 cc of 5% Evans Blue Dye (T-1824). It was found that within 5 minutes after painting with bromobenzene, the painted area became deeply stained with dye. The local accumulation of dye was sharply confined to the painted area and outlined almost exactly the extent of application of the swab. The skin remained deeply stained for about 12 hours, after which fading occurred gradually.

Before testing the effect of bromobenzene on the permeability of the vessels in skin tissue which had been prepared for the Shwartzman phenomenon, it was of interest to determine whether such tissues showed any increase in permeability to Evans Blue dye. Accordingly, rabbits were given intradermal injections of meningococcal toxin and at various intervals thereafter were given an intravenous injection of 5 cc of 5% Evans Blue. It was observed that the prepared skin did not become stained at any time up until the actual precipitation of hemorrhage following the intravenous injection of toxin. These results were similar to those described by Bordet,⁴ who used trypan blue dye in similar experiments.

³ Menkin, V., *Dynamics of Inflammation*. The Macmillan Co., New York. 1940.

⁴ Bordet, P., *Ann. Inst. Pasteur*, 1936, **56**, 325.

Having ascertained that skin areas with meningococcal toxin were not more permeable to circulating dye than normal rabbit skin, the effect of bromobenzene on prepared skin was determined. The results were quite different from those seen in normal skin. When bromobenzene was applied to an area which had been injected intradermally with toxin eighteen hours previously, and an intravenous injection of Evans Blue given simultaneously, no dye appeared in the prepared area. When a large surface of the abdomen of a prepared rabbit was painted, the contrast between the deep blue staining of the surrounding normal skin and the pink, unstained prepared area was striking.

These observations suggested that the vessels in a prepared skin area were actually less permeable to dye than normal vessels, or were less responsive to the permeability-enhancing property of bromobenzene. Similar results were obtained with surface applications of chlorobenzene, iodobenzene, and benzene, and to a less striking degree with chloroform and methyl salicylate; in each instance normal skin showed staining with the dye while prepared skin showed little or no staining.

Discussion. The inhibition of the Schwartzman phenomenon by the local application of bromobenzene and certain other organic solvents was a somewhat accidental finding. As a result of certain other experimental data,⁵ an hypothesis was entertained which implicated sulfhydryl-activatable tissue proteolytic enzymes, or "cathepsins", in the mechanism of damage to blood vessel walls in the Schwartzman phenomenon. Crabtree⁶ demonstrated that the application of bromobenzene caused prompt reduction in the amount of glutathione in skin tissue, presumably as the result of local detoxification of bromobenzene. Accordingly, the possibility was considered that an application of bromobenzene to a skin area which had been prepared for the Schwartzman phenomenon might inhibit the hemorrhagic reaction by interfering with the activation of tissue protease. The finding that

inhibition of the phenomenon was, in fact, produced by bromobenzene does not indicate that this is the mechanism by which the inhibition took place, in view of the observation that other unrelated substances such as chloroform and methyl salicylate caused a similar effect.

The capacity of bromobenzene, as well as the other solvents tested, to bring about a local increase in the permeability of normal skin to circulating Evans Blue dye offers a second possible explanation for the inhibitory action of these substances on the Schwartzman phenomenon. Although no increased permeability was demonstrable in prepared skin tissue by the dye method, it is reasonable to assume that a similar change, although less in degree, may have occurred. Under such a circumstance, inhibition of the Schwartzman phenomenon may have been caused by the advent of an inhibitory substance from the blood into the prepared area, or by the departure of a damaging substance from the prepared area.

The third possible explanation to be considered is that the inhibition is the result of a wholly non-specific damaging action of bromobenzene on the skin blood vessels, interfering with their reactivity to a variety of other stimuli.

The question of the permeability of skin capillaries is of importance in considering the pathogenesis of the Schwartzman phenomenon. If these vessels were more permeable than normal, as is reported to be the case in other varieties of inflammatory tissue,³ the accumulation of toxic material from the circulating blood would require consideration as a possible mechanism for the tissue damage,^{3,7} even though the likelihood of bacterial toxin having a direct hemorrhagic effect in such small concentrations seems remote. The studies of blood vessel permeability with intravenously injected Evans Blue dye indicate that the prepared skin is not more permeable to this material, and the results obtained with bromobenzene suggest that the vessels are less permeable, at least to this type of permeability-enhancing stimulus.

⁵ Thomas, L., and Stetson, C. A., in press.

⁶ Crabtree, H. G., *Cancer Research*, 1941, 4, 688.

⁷ Moritz, A. R., *J. Exp. Med.*, 1937, 66, 693.

Other studies in this laboratory⁵ have shown that skin tissue prepared for the Shwartzman phenomenon exhibits an abnormal degree of aerobic glycolysis and lactic acid accumulation. Whether this alteration is related to an impairment in the permeability of vessels in prepared skin is a subject for further investigation. It is of interest to note that the application of bromobenzene to prepared skin had no demonstrable effect on the degree of aerobic glycolysis exhibited by such tissue.⁵

Summary. Complete inhibition of the Shwartzman phenomenon was produced by a single application of bromobenzene to the surface of prepared skin areas at any time during the 20 hours after the intradermal injection of bacterial toxin. Similar results were obtained with other benzene derivatives, and,

less constantly, with chloroform and methyl salicylate.

Areas of rabbit skin which were prepared by the intradermal injection of meningococcal toxin showed no increase in permeability to Evans Blue dye, when the dye was injected intravenously.

A single application of bromobenzene to the surface of normal rabbit skin resulted in the prompt appearance of intravenously injected Evans Blue dye in the painted area. In contrast, little or no dye appeared in painted areas which had previously been injected with meningococcal toxin. Similar results were obtained when skin was painted with other benzene derivatives, chloroform and methyl salicylate.

The possible bearing of these observations on the problem of the mechanism of the Shwartzman phenomenon is discussed.

⁵ Unpublished observation.

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Influence of Dinitrophenol on Body Temperature Threshold for Thermal Polypnea.*

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The capacity of dinitrophenol (DNP) to cause hyperthermia in animals through accelerating heat production is well known. In animals at low environmental temperatures, however, it reduces body temperature and total heat production,¹ apparently through reducing the activity of the cold defense mechanism, as shown by depression of shivering.² In discussing the mechanism of this inactivation of cold defense, Hall, Crismon and Chamberlin² suggested two possibilities:

(a) DNP might depress the centers involved in cold defense by a nonspecific toxic action similar to that by which it depresses other nervous processes such as spontaneous running activity;³ (b) DNP, which increases the oxygen consumption of cerebral cortical tissue in concentrations likely to be attained when ordinary doses are injected,⁴ might accelerate the metabolic activity of the temperature regulating centers, so causing the centers to behave as if heated which would reduce or abolish shivering. If the latter were the case, one would expect from the known reciprocal relationship of the cold and heat defense mechanisms that, when the body temperature

* This work was done under a contract between the Air Materiel Command, Wright Field, and Stanford University.

¹ Giaja, J., and Dimitrijevic, I. N., *Arch. internat. Pharm. Therap.*, 1933, **45**, 342; Tainter, M. L., *J. Pharm. Exp. Therap.*, 1934, **51**, 45.

² Hall, V. E., Crismon, J. M., and Chamberlin, P. E., *J. Pharm. Exp. Therap.*, 1937, **59**, 193.

³ Hall, V. E., and Lindsay, M., *J. Pharm. Exp. Therap.*, 1934, **51**, 430.

⁴ Fuhrman, F. A., and Field, J., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 504.

TABLE I.
Effect of Dinitrophenol and of Salt Solution on Threshold for Thermal Polypnea of Rabbits.

Rabbit No.	Normal threshold °C	DNP threshold °C	Change in threshold °C
A. Dinitrophenol (20 mg per kg)			
1A	39.4	41.0	+1.6
1B	40.1	41.0	+0.9
2A	40.4	41.9	+1.5
2B	40.9	40.7	-0.2
2C	40.9	41.4	+0.5
3A	40.0	40.9	+0.9
3B	39.5	40.2	+0.7
4A	40.3	41.7	+1.4
4B	40.1	41.6	+1.5
4C	40.1	41.0	+0.9
Avg	40.2	41.1	+0.9
B. Controls with physiological salt solution			
Rabbit No.	Normal threshold	Threshold after saline	Change in threshold
5A	39.5	39.1	-0.4
5B	39.0	39.0	0.0
5C	39.1	39.6	+0.5
6A	39.0	38.1	-0.9
6B	38.4	38.3	-0.1
Avg	39.0	38.8	-0.2

is raised, processes accomplishing heat loss should be more readily activated under the influence of DNP than in its absence. Among other effects, DNP should (at constant environmental temperature) reduce the body temperature level at which thermal polypnea appears. The experiments described here were designed to test this possibility.

Albino rabbits weighing between 2 and 3 kg were placed in a box in which the air temperature was maintained at $30^{\circ}\text{C} \pm 2^{\circ}$. This environmental temperature is higher than that at which the rabbit can maintain a constant body temperature. Accordingly a progressive rise in body temperature occurred, and with it a rise in respiratory rate. When the latter reached 300 per min., the rectal temperature was measured with a mercury thermometer. This value was designated as the normal threshold for thermal polypnea.

The rabbits were then placed in a refrigerator at about 10°C per 45 to 60 min. Their rectal temperatures were reduced by this procedure to values well below the normal threshold for thermal polypnea. A dose of 20 mg per kg of the sodium salt of 2,4-dinitrophenol was then injected intramuscularly and the rabbits returned to the box at 30°C . As their rectal temperatures rose under the com-

bined action of the high environmental temperature and the calorogenic action of the drug, the respiratory rate accelerated. The rectal temperature at which it again reached 300 per min. was determined and designated as the DNP threshold for thermal polypnea.

The results of 10 such experiments are shown in Table I. With one exception, the DNP thresholds for thermal polypnea are greater than the corresponding normal thresholds. The average change in threshold after DNP was $+0.9^{\circ}\text{C}$. The chances that these results could have occurred by chance have been calculated to be less than one in one thousand.

A second series of 5 rabbits was treated in the same manner except that a volume of physiological salt solution equal to the volume of DNP solution was injected. The results, also given in Table I, show an average reduction of the threshold determined after saline injection amounting to 0.2°C . This finding rules out the possibility that, in the DNP series, the elevated threshold might be due to factors other than those attributable to DNP itself.

In view of the possibility that the dose of 20 mg per kg might have toxic effects not shown by lower doses, 3 rabbits subjected to

the procedure described above, were given doses of 10 mg per kg. The rectal temperatures at which respiratory rates of 200 per min. were attained before and after DNP respectively were: 39.8 and 40.2; 39.8 and 40.0, and 39.6 and 39.8°C, the threshold being increased by an average of 0.3°C. This difference is smaller than but in the same direction as the difference obtained with the larger dose of DNP.

Conclusion. We may conclude that DNP

in doses of 10 and 20 mg per kg, although it does not prevent the occurrence of thermal polypnea, does significantly increase the rectal temperature level at which such polypnea appears. It accordingly appears that DNP does not sensitize the heat defense mechanism as would be required by the second hypothesis stated above. Rather it suggests that DNP depresses both heat and cold defense mechanisms by a nonspecific toxic action.

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Effect of Insulin on Rate of Metabolism of Ethyl Alcohol.

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A review of the literature shows that the great majority of workers who have investigated the problem found that insulin in adequate dosage was effective in accelerating the rate of metabolism of alcohol. Thus Supniewski¹ showed that 1 unit of insulin per kg administered to rabbits about doubled the rate at which alcohol disappeared from the blood. Newman and Cutting² gave the same dose of insulin to 2 human subjects after administration of alcohol intravenously and found a 50% increase in rate of metabolism. Serianni³ found a similar increase in man after a dose of 20 units of insulin per individual. Widmark⁴ showed that 0.85 unit per kg in the dog accelerated the rate of alcohol metabolism, particularly in dogs with an initially low rate of metabolism of alcohol, but that the effect varied in intensity from dog to dog and in the same dog from day to day. Clark and his associates^{5,6} gave doses of 1 and 2 units

per kg to dogs and found a significant increase in rate of disappearance of alcohol from the blood.

Gregory, Ewing and Duff-White,⁷ on the contrary, reported that "there is no evidence from 24 experiments in 6 dogs that insulin, glucose, or insulin plus glucose increases the rate of metabolism of ethyl alcohol." They refer to certain reports in the literature in support of this finding. Thus Herschfelder and Maxwell⁸ concluded that insulin in doses up to 3 units per kg did not expedite the disappearance of symptoms of intoxication in the rabbit. When one considers that such doses of insulin might well in themselves induce a state similar to alcoholic intoxication in its symptomatology, one is not justified in drawing any conclusions from these experiments regarding the effect of insulin on rate of alcohol metabolism. Fleming and Rey-

⁵ Clark, B., and Morrissey, R., *Am. J. Physiol.*, 1938, **123**, 37.

⁶ Clark, B., Morrissey, R., Fazekas, J., and Welch, C., *Quart. J. Studies Alc.*, 1941, **1**, 663.

⁷ Gregory, R., Ewing, P., and Duff-White, V., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 206.

⁸ Herschfelder, A. D., and Maxwell, H. C., *Am. J. Physiol.*, 1924, **70**, 520.

¹ Supniewski, J., *J. Biol. Chem.*, 1926, **70**, 13.

² Newman, H. W., and Cutting, W. C., *J. Clin. Invest.*, 1935, **14**, 945.

³ Serianni, E., *R. C. Accad. Lincei*, 1935, **21**, 394.

⁴ Widmark, E. M. P., *Biochem. Z.*, 1935, **282**, 79.

TABLE I.
Effect of Dinitrophenol and of Salt Solution on Threshold for Thermal Polypnea of Rabbits.

Rabbit No.	Normal threshold °C	DNP threshold °C	Change in threshold °C
A. Dinitrophenol (20 mg per kg)			
1A	39.4	41.0	+1.6
1B	40.1	41.0	+0.9
2A	40.4	41.9	+1.5
2B	40.9	40.7	-0.2
2C	40.9	41.4	+0.5
3A	40.0	40.9	+0.9
3B	39.5	40.2	+0.7
4A	40.3	41.7	+1.4
4B	40.1	41.6	+1.5
4C	40.1	41.0	+0.9
Avg	40.2	41.1	+0.9
B. Controls with physiological salt solution			
Rabbit No.	Normal threshold	Threshold after saline	Change in threshold
5A	39.5	39.1	-0.4
5B	39.0	39.0	0.0
5C	39.1	39.6	+0.5
6A	39.0	38.1	-0.9
6B	38.4	38.3	-0.1
Avg	39.0	38.8	-0.2

is raised, processes accomplishing heat loss should be more readily activated under the influence of DNP than in its absence. Among other effects, DNP should (at constant environmental temperature) reduce the body temperature level at which thermal polypnea appears. The experiments described here were designed to test this possibility.

Albino rabbits weighing between 2 and 3 kg were placed in a box in which the air temperature was maintained at $30^{\circ}\text{C} \pm 2^{\circ}$. This environmental temperature is higher than that at which the rabbit can maintain a constant body temperature. Accordingly a progressive rise in body temperature occurred, and with it a rise in respiratory rate. When the latter reached 300 per min., the rectal temperature was measured with a mercury thermometer. This value was designated as the normal threshold for thermal polypnea.

The rabbits were then placed in a refrigerator at about 10°C per 45 to 60 min. Their rectal temperatures were reduced by this procedure to values well below the normal threshold for thermal polypnea. A dose of 20 mg per kg of the sodium salt of 2,4-dinitrophenol was then injected intramuscularly and the rabbits returned to the box at 30°C . As their rectal temperatures rose under the com-

bined action of the high environmental temperature and the calorogenic action of the drug, the respiratory rate accelerated. The rectal temperature at which it again reached 300 per min. was determined and designated as the DNP threshold for thermal polypnea.

The results of 10 such experiments are shown in Table I. With one exception, the DNP thresholds for thermal polypnea are greater than the corresponding normal thresholds. The average change in threshold after DNP was $+0.9^{\circ}\text{C}$. The chances that these results could have occurred by chance have been calculated to be less than one in one thousand.

A second series of 5 rabbits was treated in the same manner except that a volume of physiological salt solution equal to the volume of DNP solution was injected. The results, also given in Table I, show an average reduction of the threshold determined after saline injection amounting to 0.2°C . This finding rules out the possibility that, in the DNP series, the elevated threshold might be due to factors other than those attributable to DNP itself.

In view of the possibility that the dose of 20 mg per kg might have toxic effects not shown by lower doses, 3 rabbits subjected to

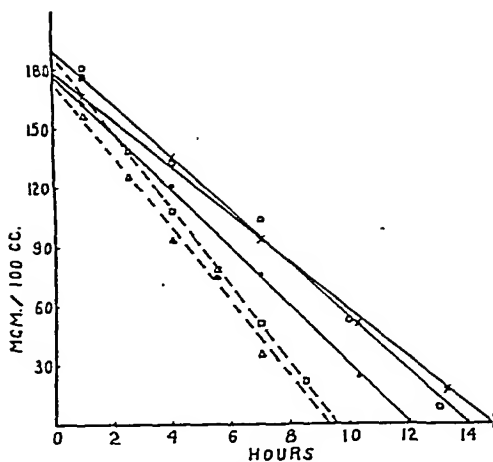


FIG. 1.

Blood alcohol concentrations after intravenous injection of 1.5 g of alcohol per kg in a dog. Three control injections are represented by crosses, open circles, and dots. Two injections with insulin 1 unit per kg are shown with open squares and open triangles. The best fitting lines between these points are extrapolated to the base line to estimate the time of disappearance of alcohol from the blood stream.

on each dog. Then the same procedure was repeated after administration of a single dose of 1 unit of regular insulin per kg, and in the animal which showed the greatest acceleration the insulin run was repeated so that we could be doubly sure of the results. The

results are presented in Table I, and those in the dog which had the 2 trials with insulin are shown graphically in Fig. 1. It is seen that 2 of the 3 animals responded to this dose of insulin by an increase in rate of alcohol metabolism of approximately 50%, while the slight increase in the third animal, although deviating from the average of the control values by a considerable margin over the range of these values, was far less striking. The same procedure was repeated with two other dogs, with the exception that the dose of insulin was reduced to 0.5 unit per kg. In neither animal did this smaller dosage of insulin produce any acceleration of alcohol metabolism.

Summary. Insulin in a dose of 1 unit per kg was found to be variably effective in accelerating the rate of alcohol metabolism, the effect being striking in 2 dogs, much less in another. This is in accord with the variable results reported by Widmark.⁴ Half this dose was entirely ineffective in 2 dogs. The failure of Gregory and coworkers⁷ to demonstrate this accelerating action of insulin in adequate dosage must be due to the possibility that their 6 dogs fell, by chance, into the group of animals which does not show a striking acceleration with insulin.

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Prolongation of Pseudopregnancy by Deciduomata in the Rat.*

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Astwood and Greep¹ have stated that the presence of deciduomata in the rat does not prolong the diestrus of pseudopregnancy. Ershoff and Deuel,² however, have presented

data showing pseudopregnancy in the rat to be prolonged as much as 7 days by production of deciduomata. More recently Kamell and Atkinson³ reported that deciduomata do not prolong pseudopregnancy in the mouse. The following data confirm those of Ershoff and Deuel.

* Supported in part by a grant from Ciba Pharmaceutical Products, Inc.

¹ Astwood, E. B., and Greep, R. O., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 713.

² Ershoff, B. H., and Deuel, H. J., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 167.

³ Kamell, S. A., and Atkinson, W. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 415.

nolds⁹ gave one human subject a dose of 10 units of insulin and found no effect on the rate of disappearance of alcohol injected intravenously. Here the dosage is conspicuously lower than that employed by those workers cited above who found an effect from insulin. The work of Goldfarb, Bowman and Parker¹⁰ can hardly be classed as supportive, since they found that while neither insulin nor glucose alone expedited the removal of alcohol from moderately intoxicated patients, a combination of the two was effective. Neither can the unelaborated statement of Mirsky and Nelson¹¹ that in dogs "treatment with insulin and glucose" had little effect on the rate of alcohol metabolism be very heavily weighted as evidence. Thus it is seen that the evidence in the literature referred to by Gregory and coworkers is not impressive. However, a short communication by Harger and Hulpieu¹² is less easily disposed of. These workers found that the rate of fall of alcohol concentration in dogs after gastric administration of doses from 1.5 to 3.0 g per kg was not changed by the administration of 1 unit of insulin per kg, alone or with glucose. Gregory and coworkers would, however, object to this work on the basis that the alcohol was given by mouth, since they reported that after gastric administration the blood alcohol levels were so variable, and disappearance curves so irregular, that this was an unsatisfactory method of giving alcohol for this type of investigation.

For this reason, they⁷ administered the alcohol intravenously, and followed the concentration in the blood until it approached zero. They published one graph which shows the curves of blood alcohol concentration in one dog after a control dose of 2.0 cc per kg of alcohol alone, alcohol and glucose, alcohol and insulin 1 unit per kg, and finally alcohol and

combination of insulin and glucose. All of the curves reached the base line in from 11 to 12 hours, so that there is no evidence in this dog of acceleration by insulin. The authors stated that the results in the other 5 dogs were "practically identical", but did not publish the data on which they based this statement.

In view of the conflict of these results with most of the reported work, including our own work in man where the method of procedure was practically identical, it was felt that further investigation of the problem, adhering closely to the methodology of Gregory and coworkers, was indicated.

To this end, alcohol was administered intravenously to each of three dogs in a dose approximating 2 cc per kg, and the blood alcohol concentration followed with serial samples until it approached zero. Analyses for alcohol were carried out according to the method of Newman.¹³ Extrapolation of the best-fitting line between these points to the base line was used to estimate the time required for disappearance of alcohol, and from this it was a simple matter to calculate the average rate of metabolism in mg per kg per hour. In order to establish fully the range of variation in this rate in a given dog from day to day, 3 controls with alcohol alone were run

TABLE I.
Rate of Metabolism of Alcohol With and Without Insulin in Dosage of 1 Unit per Kg Body Weight.

Dog	Wt kg	Dose alcohol g/kg	Rate of metabolism	
			Control	Insulin
1	23	1.5	102	
		1.5	98	
		1.5	103	
		1.5		143
2	21	1.5	123	
		1.5	102	
		1.5	107	
		1.5		158
5	10.5	1.43	102	
		1.43	104	
		1.43	106	
		1.43		112

⁹ Fleming, R., and Reynolds, D., *J. Pharmacol. and Exp. Therap.*, 1935, **54**, 236.

¹⁰ Goldfarb, W., Bowman, K. M., and Parker, S., *J. Clin. Invest.*, 1939, **18**, 581.

¹¹ Mirsky, I. A., and Nelson, N., *Am. J. Physiol.*, 1939, **127**, 308.

¹² Harger, R. N., and Hulpieu, H. R., *J. Pharmacol. and Exp. Therap.*, 1935, **54**, 145.

¹³ Newman, H. W., *J. Pharmacol. and Exp. Therap.*, 1936, **50**, 278.

Effect of Cardiac Drugs on the Phosphorylated Intermediates of the Rat.*

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Reports¹⁻³ have appeared in the literature on the effects of cardiac drugs on glycogen metabolism of laboratory animals. Several studies on the effect of cardiac drugs on the concentration of certain acid-soluble phosphorus compounds have been reported. These findings have been summarized in a recent review⁴ on the biochemistry and pharmacology of the heart. Wollenberger⁵ concluded from his studies on dog heart-lung preparations that the primary action of cardiac glycosides must be concerned with a phase of myocardial contraction other than the generation of utilizable chemical energy. On a series of perfusion studies of the isolated hearts of cats, rabbits, and rats, with digitoxin, Sjoerdsma *et al.*⁶ reported that the appearance of creatinine in the perfusion fluid is not altered. However, no systematic analysis of the phosphorylated intermediates of glycolysis has been reported. The purpose of the present investigation was to measure the concentration of a number of phosphorylated intermediates of glycolysis under various conditions of treatment with cardiac drugs.

Experimental. Normal Sprague-Dawley

* This work was supported by grants from the Life Insurance Medical Research Fund. The digitoxin used in these experiments was kindly supplied by Dr. K. K. Chen of the Lilly Research Laboratories, Indianapolis, Ind.

¹ Liebig, H., *Arch. exp. Path. Pharmacol.*, 1940, **196**, 137.

² Cherkes, A. I., *Acta Med., U.R.S.S.*, 1940, **3**, 155.

³ Bomskov, C., *Arch. exp. Path. Pharmacol.*, 1941, **108**, 232.

⁴ Chen, G., and Geiling, E. M. K., *Schweiz. Med. Wochenschrift*, Basle, 1947.

⁵ Wollenberger, A., *Am. J. Physiol.*, 1947, **150**, 733.

⁶ Sjoerdsma, A., Kun, E., Schueler, F. W., and DeValle, J. E., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 144.

rats of approximately 250 g were digitalized intraperitoneally by the daily administration of 12 mg/kg of crystalline digitoxin in propylene glycol for 3 days. Control animals received equivalent amounts of the solvent throughout the digitalization period. Following a fasting period of 24 to 36 hours, the animals were anesthetized by the intraperitoneal injection of 45 mg/kg of sodium pentobarbital, and decapitated immediately after light anesthesia. The tissues were quickly blotted on filter paper and frozen between slabs of dry ice, weighed, and the phosphorus fractions then measured according to the methods outlined by LePage and Umbreit.⁷ Glycogen in normal and digitalized rat heart was determined on separate samples of tissues by the method of Good *et al.*⁸ The method of Folin and Malmros⁹ was used for measuring the glucose after hydrolysis of glycogen.

None of the digitalized animals elicited any symptoms of over-digitalization; such as tremors or convulsions. The data presented in Table I indicate that digitoxin produced no marked alteration in the distribution of the acid-soluble phosphorylated intermediates of the rat heart. Although reports have appeared in the literature on changes in the phosphocreatine content of the heart under the influence of cardiac drugs, no such effect was noticed, under the conditions of the experiment.

Results presented in Table II indicate that there was no marked difference in heart glycogen values, but that there was an increased amount of liver glycogen in the digitalized animals as compared with the controls, there

⁷ LePage, G. A., and Umbreit, W. W., *Manometric Techniques*, Minneapolis, 1945.

⁸ Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 484.

⁹ Folin, C., and Malmros, H. J., *J. Biol. Chem.*, 1929, **83**, 115.

TABLE I.
Prolongation of Pseudopregnancy by Deciduomata.

Group	No. of rats	No. of deciduomata	Pseudopregnancy (days)	Standard error	St. error of diff.* and probability
Pseudopregnant	18	8	13.33	0.220	± 1.043 and <0.01
Pseudopregnant with deciduomata	10	8	20.60	1.287	
Pseudopregnant	11	4	18.55	1.290	± 1.340 and <0.01
deciduomata removed	17	4	14.25	0.680	

* Pooled variance. Snedecor *Statistical Methods*, University of Iowa College Press, 1947.

Methods and results. Daily vaginal smears were made from 18 female albino rats (150 to 250 g) of mixed strain. On the day of vaginal estrus (fully cornified smear) their cervixes were stimulated electrically† to induce pseudopregnancy. Vaginal smears were made until the next vaginal estrus. The first day of diestrus was considered day one and the first day of full vaginal cornification the last day of pseudopregnancy. The average length of pseudopregnancy was found to be 13.33 days. (Table I).

Ten similarly prepared rats were subjected to laparotomy on the fourth day of pseudopregnancy. Both uteri were stimulated by passing a suture transversely through the walls and lumen at 4 points in each, tying loosely and leaving the suture *in situ* as a marker. On the 8th day of pseudopregnancy at a second laparotomy, the number of deciduomata formed in each rat was determined. Daily smearing was continued as before. Each rat responded with eight deciduomata, the maximum number possible. The average length of pseudopregnancy in this group was 20.60 days (see Table I). The difference of 7.27 days is highly significant statistically.

To determine whether some factor other than the presence of deciduomata was respon-

sible for prolongation of diestrus an additional 28 pseudopregnant rats were treated as in the previous experiment except that at laparotomy on the 4th day *only one uterus (the right) was stimulated in 4 places*. On the eighth day the number of deciduomata formed in each was noted. Again all 28 rats responded with the maximum number, 4 deciduomata. The right uterus containing the deciduomata was then removed in 17 of the 28 rats. The average length of pseudopregnancy in the 17 rats in which the deciduomata were removed was 14.25 days (not significantly different from the length of normal pseudopregnancy). The length of pseudopregnancy in the 11 rats in whom the deciduomata were not removed was 18.55 days (not significantly different from the length of pseudopregnancy in the rats with 8 deciduomata).

As may be seen in Table I the difference of 4.30 days is statistically significant.

Our data demonstrate that the presence of deciduomata in the pseudopregnant rat significantly prolongs the period of diestrus. The mechanism involved is not known but the evidence presented here is not adequate to prove that it is a specific effect of the deciduoma, *per se*.

Summary. The presence of deciduomata prolongs the length of pseudopregnancy in the rat an average of 7.27 days beyond the normal duration of 13.33 days.

† A bipolar electrode attached to a Harvard inductorium was inserted in the vagina.

TABLE III.

Effect of Digitoxin on Synthesis of Liver Glycogen in Rats Given 5 mg/kg Digitoxin in Propylene Glycol for 3 days; Fasted 24 Hours; No Further Injection of Drug Given During Glycogenesis; No Convulsions or Tremors.

Glucose schedule	Control				Digitalized			
	Exp. No.	Rat No.	% glycogen	Mean	Exp. No.	Rat No.	% glycogen	Mean
No Glucose	I	1	0.59		I	9	0.95	
		2	0.63	0.61		10	0.85	0.90
	II	17	0.19		II	25	1.02	
		18	0.41	0.30		26	0.99	1.00
0.15 g/hr for 2 hrs before death	I	3	1.22		I	11	2.36	
		4	1.27	1.24		12	2.52	2.44
	II	19	0.48		II	27	1.86	
		20	0.61	0.54		28	1.43	1.64
0.15 g/hr for 4 hrs before death	I	5	2.09		I	13	2.81	
		6	2.03	2.06		14	2.71	2.76
	II	21	1.03		II	29	1.96	
		22	1.18	1.10		30	2.80	2.38
0.15 g/hr for 6 hrs before death	I	7	2.05		I	15	2.86	
		8	2.13	2.09		16	2.84	2.85
	II	23	1.06		II	31	2.86	
		24	1.32	1.19		32	2.43	2.64

TABLE IV.

Effect of Digitoxin on Synthesis of Liver Glycogen in Over-Digitalized Rats Given 4 mg/kg Digitoxin in Propylene Glycol for 3 days; Fasted 24 Hours; Additional Injection of 5 mg/kg Digitoxin Given at 0 Hour.

Glucose schedule	Control			Digitalized		
	Rat No.	% glycogen	Mean	Rat No.	% glycogen	Mean
No glucose	34	0.31		40*	0.21	
	35	0.29	0.30	41*	0.17	0.19
0.15 g/hr for 2 hrs before death	36	1.01		42†	0.04	—
	37	0.86	0.93	43*	0.29	—
0.15 g/hr for 4 hrs before death	38	1.83		44†	Died	—
	39	1.73	1.78	45†	0.02	—

* Occasional tremors.

† Tremors and convulsions.

in digitalized animals. There was no additional injection of the drug during the 6 hour period of glucose injection. None of the digitalized animals showed any tremors, convulsions, or other toxic symptoms. Difference in normal values of Experiment I and II are probably due to differences in amount of previously ingested food, prior to fasting.

The results given in Table IV show the effect of digitoxin on glycogen synthesis by the liver after administration of glucose to

fasted over-digitalized rats. A group of six rats which had been digitalized for 3 days by the daily administration of 4 mg/kg of crystalline digitoxin in propylene glycol was given an additional injection of 5 mg/kg of digitoxin in propylene glycol intraperitoneally at the start of the glucose injection period. The controls received an equivalent amount of the solvent. Both groups were fasted for 24 hours. It can be seen from the data that there was a decreased amount of

TABLE I.
Distribution of Acid-Soluble Phosphorus Compounds in the Heart of Normal and Digitalized Rats.

Phosphorylated Intermediates*	Normal		Digitalized	
A. Analysis on T.C.A. Extracts:				
1. "True" Inorganic Phosphorus	993		1100	
2. Phosphocreatine Phosphorus	262		293	
3. Phosphopyruvic	1450		1242	
4. Total Phosphorus	3110		3184	
	Barium-Soluble		Barium-Insoluble	
	Normal	Drug	Normal	Drug
B. Analysis of Fractions:				
1. Inorganic Phosphorus	239	245	1232	1326
2. ATP 7 min Hydrolyzable			229	200
3. Total Phosphorus	877	1180	1693	2155
4. Fructose	17	18		
5. Fructose 1-6 Diphosphate			102	97

* Expressed as micromoles/100 g.

TABLE II.
Effect of Digitoxin on Liver and Heart Glycogen of Rat Given 12 mg/kg Digitoxin in Propylene Glycol for 3 Days, Fasted 24-36 Hours, and Anesthetized with 45 mg/kg Sodium Pentobarbital.

Tissue	Control			Digitalized		
	Rat No.	% glycogen	Mean	Rat No.	% glycogen	Mean
Heart	1	0.34	0.27	4	0.30	0.30
	2	0.27		5	0.29	
	3	0.21		6	0.31	
Liver	1	1.96	1.85	4	2.06	2.39
	2	1.88		5	2.32	
	3	1.71		6	2.81	

being 2.39% glycogen in the former and 1.85% in the latter. These values suggested the possibility that digitoxin may exert a protective action on liver glycogen by decreasing the rate of glycogenolysis and experiments were, therefore, conducted to measure this possibility. Samples of liver tissue from both digitalized and normal animals were allowed to stand at room temperatures for periods of 2, 5, and 8 minutes prior to freezing with dry ice. Results indicated that there was no difference in the rate of breakdown of glycogen in the excised liver tissue in normal and digitoxin treated rats. It should be noted that these and subsequent experiments, in which the liver has been removed from its normal environment, do not take into consideration the possible influence of epinephrine or insulin on glycolysis or glycogenesis *in situ*.

Inasmuch as Yorimitsu¹⁰ had demonstrated that digitalis preparations promoted resynthesis of glycogen from lactic acid, experiments were conducted to ascertain the effect of digitoxin on the synthesis of liver glycogen from glucose in rat liver, using the method as outlined by DuBois *et al.*¹¹ Normal Sprague-Dawley rats of approximately 250 g were digitalized intraperitoneally with 5 mg/kg of crystalline digitoxin in propylene glycol for 3 days. Controls received equivalent amounts of the solvent during the digitalization. The experiments were begun following a fasting period of 24 to 36 hours. Data given in Table III show that the initial glycogen values were higher in the digitalized animals, and there was an indication of an increased amount of glycogenesis from injected glucose

¹⁰ Yorimitsu, T., *Osaka Igakkai Zasshi*, 1940, 39, 1381.

¹¹ DuBois, K. P., Holm, L. W., and Doyle, W. L., *Proc. Soc. Exp. Biol. and Med.*, 1946, 61, 102.

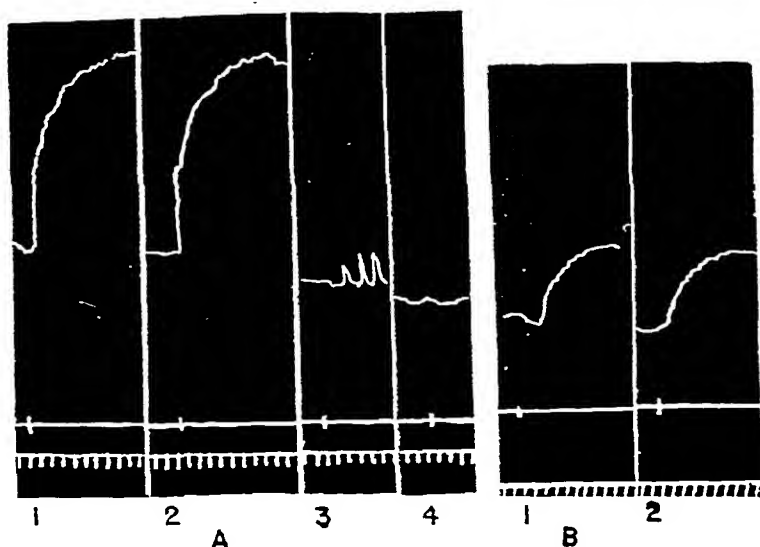


FIG. 1.

Contraction of the isolated guinea pig uterus. A, 1, 0.005 of 1.5 U Oxytocin; 2, 0.005 of 1.5 U Oxytocin + 5 mg fibrinolysin; 3, 0.005 of 1.5 U Oxytocin + 0.5 cc serum from patient in 9th month of pregnancy + 5 mg fibrinolysin; 4, 0.005 of 1.5 U Oxytocin + 0.5 cc pregnant woman serum. B, 1, 0.005 of 1.5 U Oxytocin + 20 mg fibrinolysin + 5 mg glutathione; 2, 0.005 of 1.5 U Oxytocin + 20 mg fibrinolysin. In A the incubation period lasted 24 hours, and 30 hours in B.

oxytocin. However hypertensinogen is not attacked by this enzyme, and after the addition of fibrinolysin it is still capable of producing hypertensin when renin is added.

Material and method. Three different samples of fibrinolysin were used, one prepared by us from ox plasma, activated with chloroform by the Loomis technic,⁸ and two others obtained by fractionation of human plasma prepared by the Physical Chemical Department of Harvard Medical School.

The sample with which we did most of the experiments, (Squibb III-3-1) contained approximately 0.5 U per mg, according to Loomis. The substrates were: a) purified extract of neurohypophysis which contained 10 U.v. of oxytocin and 10 U of vasopressin per cc and b) human and horse hypertensinogen. In order to study the oxytocin fibrinolysin reaction a guinea pig uterus suspended in oxygenated Tyrode's solution was used. The blood pressure of the cat was employed in order to determine the inactivation of vaso-

pressin by the enzyme. This same test was used to titrate the amount of hypertensin formed by the interaction of hypertensinogen with the enzymes:

Effect of fibrinolysin upon oxytocin. Different amounts of fibrinolysin: 2, 5 and 10 mg in 0.9% NaCl were made to act *in vitro* upon 2 U.v. of oxytocin; the solution was adjusted to pH 7.3 by means of a solution of sodium phosphate (0.2 normal). The total volume was brought up to 2.5 cc. The control contained 2 U.v. of oxytocin without the enzyme. The tubes were incubated at 37°C from 4 to 72 hours. When the incubation time exceeded 4 hours a drop of sodium merthiolate (Lilly) was added to each tube, as a bactericidal agent. As seen in Fig. 1 the oxytocic activity remains unchanged even after an incubation of 72 hours in the presence of 10 U of fibrinolysin, therefore it is quite clear that oxytocin is not hydrolyzed by fibrinolysin.

In experiments with pregnant woman serum it was proved fibrinolysin does not increase the destruction of oxytocin by the

⁸Loomis, C. E., George, C. H., and Ryder, A., *Arch. Bioch.*, 1947, 1, 12.

liver glycogen in overdigitalized animals, the per cent of glycogen decreasing somewhat as the animals approached death. These results seem to indicate that increased glycogenesis occurred in the liver of rats given non-toxic doses of digitoxin, while toxic doses of the drug resulted in a decrease in the amount of liver glycogen in rats in which symptoms of over-digitalization are manifested.

Summary. There was no marked difference in the distribution of the acid-soluble phosphorylated intermediates of glycolysis in the heart muscle of normal and digitoxin-poisoned rats. Small differences in the glycogen values of heart values were noted, but there was an increased amount of liver gly-

cogen in the digitalized animals as compared with the controls as indicated by an average of 2.39% of glycogen in the former and 1.85% in the liver of the latter. Neither was there any difference in the rate of breakdown of glycogen in the excised liver tissue in normal and digitoxin treated rats. Increased glycogenesis occurred in the liver of rats given non-toxic doses of digitoxin while toxic doses of the drug resulted in a decrease in the amount of liver glycogen in rats in which symptoms of over-digitalization were manifested.

The author wishes to express his appreciation to Dr. K. P. DuBois and to Dr. E. M. K. Geiling of the Department of Pharmacology for their interest and helpful advice in this problem.

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Effect of Fibrinolysin upon Oxytocin, Vasopressin and Hypertensinogen.

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Plasma fibrinolysin has been considered a tryptase. Its proteolytic effect may be observed not only in the dissolution of fibrin but also in the digestion of fibrinogen, gelatin and casein, which is shown by the production of non-protein nitrogen when it acts upon these substrates (Tagnon *et al.*;¹ Kaplan *et al.*;² Christiensen,³ Ferguson,⁴ Shinowara,⁵). Nevertheless fibrinolysin does not inactivate hypertensin (Croxatto and Badia,⁶) a substrate which is attacked by all proteolytic enzymes tested up to now, including crystallized pancreatic trypsin (Croxatto⁷).

As fibrinolysin may appear in the blood stream in its active form, an experimental study was carried out by observing its effect upon the hormones of the neurohypophysis-oxytocin and vasopressin- and upon hypertensinogen, in a similar way to that used before for trypsin. This also has the advantage of permitting a more detailed comparison between the effects of fibrinolysin and pancreatic trypsin. It has already been established that the latter is capable of inactivating hypertensin and vasopressin but not oxytocin (Croxatto⁷).

Hypertensinogen is attacked by trypsin but without the formation of hypertensin. Moreover after this enzyme has been used the substrate is incapable of producing hypertensin even when renin is added.

The results published in this paper show that fibrinolysin, just as trypsin, destroys the pressor effect of vasopressin but does not, under the same conditions, alter the effect of

¹ Tagnon, H. J., Davidson, C. S., and Taylor, H. L., *J. Clin. Invest.*, 1942, **21**, 525.

² Kaplan, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 533.

³ Christiensen, R. L., *J. Gen. Physiol.*, 1945, **28**, 363.

⁴ Ferguson, J. H., *Am. J. Med.*, 1947, **3**, 67.

⁵ Shinowara, J. G., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 456.

⁶ Croxatto, H., Badia, W., and Croxatto, R., *Bol. Soc. Biol.*, Santiago, 1948, **5**, 82.

⁷ Croxatto, H., *Revista de Medicina Aliment.*, 1943, **5**, 259.



FIG. 3.

Arterial blood pressure of the cat. 1, $\frac{1}{2}$ of 40 cc hypertensinogen at pH 6.8 + 3 cc renin, incubated during 10 minutes; 2, $\frac{1}{2}$ of 40 cc hypertensinogen at pH 6.8 + 10 mg fibrinolysin + 3 cc renin, incubated for 10 minutes; 3, $\frac{1}{2}$ of 40 cc hypertensinogen pH 6.8; 4, $\frac{1}{2}$ of 40 cc hypertensinogen + 10 mg fibrinolysin incubated for 10 minutes.

strated that the addition of ether or acetone increased the production of hypertensin by favouring the renin-hypertensinogen reaction, it appeared necessary to add these solvents to the mixture of hypertensinogen and renin. However the results were no different from those obtained without the solvents.

It was also shown that if hypertensinogen is first treated with fibrinolysin and afterwards incubated with 2 cc renin for 15 minutes, the production of hypertensin is the same as when fibrinolysin is not used. Therefore this enzyme, unlike trypsin does not modify the capacity of hypertensinogen to serve as a substrate for renin (Fig. 3).

Discussion. The effect of fibrinolysin upon the hormones of the neurohypophysis is qualitatively, comparable to that of crystallized pancreatic trypsin, however it differs from the latter by its inability to alter the hyper-

tensinogen substrate. This last result discards the possibility that fibrinolysin could have an effect comparable to renin. The proteolytic activity of fibrinolysin is limited to only certain substrates, and it does not appear to strengthen the activity of other plasma enzymes also thought to be proteolytic, such as renin, oxytocinase, hypertensinase, etc.

Even though fibrinolysin destroys vasopressin it does not have such a high vasopressinase activity as plasma during normal pregnancy. However it is possible that the increase of fibrinolytic activity observed in the plasma of pregnant women complicated by hypertensive toxemia (Willson and Munnell,¹²) may be related to a defense mechanism brought about by the capacity of this enzyme to inactive vasopressin.

It could be presumed that this effect upon the pressor hormone of the hypophysis is not due to fibrinolysin itself but to some other accompanying enzyme as yet unidentified. It cannot be hypertensinase because the addition of cysteine or glutathion to fibrinolysin does not modify its activity upon vasopressin or oxytocin (Fig. 1).

Conclusions. 10 U of fibrinolysin obtained from human and ox plasma, added to 1 U of oxytocin, does not alter the uterotonic effect of this hormone even after 72 hours of incubation at 37°C at pH 7.3. On the other hand the same preparation of fibrinolysin, under similar conditions produces a progressive destruction of vasopressin. Fibrinolysin added to human or horse hypertensinogen does not produce hypertensin, neither does it incapacitate the hypertensinogen for producing hypertensin when renin is added immediately after.

We wish to thank the Rockefeller Foundation and Dr. Edwin Cohn for the aid they have given us in the work here reported.

¹² Willson, J. H., and Munnell, E. R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 277.



FIG. 2.

Arterial blood pressure of the cat. Injections into the femoral vein. 1, 0.3 of 1.5 U vasopressin + 0.1 mg trypsin; 2, 0.3 of 1.5 U vasopressin + 20 mg fibrinolysin + 0.1 mg trypsin; 3, 0.3 of 1.5 U vasopressin + 20 mg fibrinolysin; 4, the same as in 3, but the different components were incubated separately. In all cases the incubation period lasted 24 hours at pH 7.3.

serum oxytocinase (Fig. 1).

Effect of fibrinolysin upon vasopressin. The incubation of vasopressin under similar conditions to those described above shows that fibrinolysin produces an appreciable inactivation of the pressor hormone. The titration tests were done by means of the blood pressure of the cat, anesthetized by dial. The solution was injected intravenously; in a few cases it was boiled previously. In order to discover whether fibrinolysin itself could have any influence upon the sensitivity of the cat to the pressor hormone, a mixture of fibrinolysin and vasopressin, incubated separately, was injected. Great care was taken to inject the same doses of substrate and enzyme as those which had been incubated.

As a general rule, each animal received only a few injections due to the tachyphylaxis produced by this hormone. In any case, by using a sufficiently large number of animals (3 or 4) it was clearly established that after 8 to 10 hours of incubation 10 to 20% of the vasopressin is inactivated when 10 U of fibrinolysin are used with 2 U of the pres-

or hormone. After 24 hours 40% is destroyed, and after 72 hours 80 to 100%. If crystallized trypsin is used in a dose equivalent to the fibrinolytic effect, it will be seen that 0.08 mg of this enzyme (containing 37% MgSO_4) which equals one fibrinolytic unit, destroys 0.8 U of vasopressin after 24 hours of incubation, *i. e.* the effect of 10 U of fibrinolysin, (Fig. 2).

The addition of 4 mg cysteine to 10 mg fibrinolysin, does not alter the effect of this enzyme upon vasopressin or oxytocin. This distinguishes it from blood plasma which shows a notable increase of its vasopressinase and oxytocinase effect when cysteine or glutathione are added (Croxatto, Reyes,⁹).

Effect upon hypertensinogen. Human and ox hypertensinogen were prepared as described, by precipitation with $(\text{NH}_4)_2\text{SO}_4$ at 0.5 saturation of the plasma, dialyzed with cold tap water and then filtered. The pH was brought to 7.4 by means of NaOH using a potentiometer. One hundred cc of this preparation incubated 15 minutes with 2 cc of the standard solution of pig renin gave 18 to 25 U of hypertensin. When hypertensinogen was treated with fibrinolysin for periods varying from 10 minutes to 12 hours, no pressor substance was produced. In order to investigate the possible formation of hypertensin, the mixtures of 50 cc of hypertensinogen with 1, 5, 10, and 20 mg of fibrinolysin were treated by the usual procedure for extracting hypertensin (Braun-Menéndez *et al.*¹⁰). The final extract was injected intravenously; the dose was calculated to be the equivalent of 10 to 20 cc of hypertensinogen. The results showed that the extract of hypertensinogen and fibrinolysin obtained produced exactly the same effect as the extract prepared from hypertensinogen alone, *i. e.* without hypertensin. In view of a previous paper (Croxatto and Croxatto,¹¹) which demon-

⁹ Croxatto, H., and Reyes, M., *Bol. Soc. Biol.*, Santiago, 1948, 5, 80.

¹⁰ Braun-Menéndez, E., Fasciolo, J. C., Leloir, L. F., and Muñoz, J. M., *J. Physiol.*, 1940, 133, 731.

¹¹ Croxatto, H., and Croxatto, R., *Rev. argent. de Biol.*, 1948, in press.

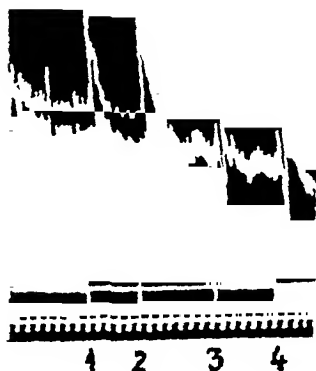


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¹¹ Croxatto, H., and Croxatto, R., *Rev. argent. de Biol.*, 1948, in press.

TABLE I.
Effect of Injection of Adrenalin on White Blood Cells of the Rat. Ten Rats Each Test.

Dose/100 g, γ	Leucocytes in %				Lymphocytes in %				t	p	Area in mg
	0'	20'	40'	60'	0'	20'	40'	60'			
	A. Normal Rats.										
1	100	70	59	60	100	64	54	54	11.6	<.001	267.2
0.2	100	71	62	60	100	64	56	49	8.38	<.001	271.6
.1	100	76	58	56	100	62	52	49	8.96	<.001	230.9
.05	100	74	63	57	100	72	62	54	5.7	<.001	199.5
.03	100	94	89	87	100	93	88	85	0.79	<.4	47.5
B. Adrenalectomized Rats.											
1	100	94	92	94	100	95	92	93			36

lin. Moreover, an attempt is made to analyze the mechanism involved.

Method. The experiments were performed on Sprague-Dawley rats (mostly adults, 150-200 g, but also some of lower weight as stated below) after a 16 hour fast. The counting of red and white blood cells including differentials followed standard procedures. It was based on duplicate or triplicate determinations. Blood samples were taken from the tail with a minimum of excitation by proper selection of tame rats and careful handling. Adrenalin was injected intraperitoneally while eserine sulphate was administered subcutaneously. The blood samples were taken at 20 minute intervals for 1 hour after the injection of adrenalin.

Results. The effect of intraperitoneal injection of adrenalin on the white blood count of rats was studied on 5 groups of animals which received this substance in quantities varying from 0.03 γ to 1 γ /100 g weight. Table I shows that the number of leucocytes and lymphocytes decreases markedly with doses from 0.05 to 1 γ /100 g whereas the effect of 0.03 γ /100 g is only slight. The individual data showing the changes in the number of lymphocytes were graphed and the area representing the fall in the number of lymphocytes was weighed. The mean value in milligrams is given under "area" in the table and the statistical significance of these changes was calculated by determination of t and P values when each of these series of experiments was compared with that performed on adrenalectomized rats (Table I, B). The latter showed only an insignificant

fall in the number of leucocytes and lymphocytes.

The table shows that the injection of adrenalin in doses varying from 0.05 to 1 γ /100 g causes a significant fall in leucocytes and lymphocytes whereas 0.03 γ /100 g adrenalin is ineffective. There seems to be little variation in the degree of leuco- and lymphopenia when the doses of adrenalin varied 20 fold.

A further group of experiments was performed on young rats averaging 40 g in weight. These animals reacted likewise with a marked leuco- and lymphopenia to the injection of 0.05 γ adrenalin per 100 g. This shows that 0.02 γ adrenalin can be readily detected by this method. Since the adrenalin content of blood is about 0.1 γ /cc (West)¹⁵ this method appears to be suitable for such studies.

Fig. 1 shows the effect of adrenalin on neutrophils as compared to lymphocytes. It is evident that increase in neutrophils and lymphopenia characterizes the effect of adrenalin on the white blood count of the rat. In spite of some differences in the time relations the experiments of Dougherty and White on the effect of the injection of the adrenotropic hormone on the leucocytes and our own on the action of adrenalin show qualitatively and quantitatively similar results.

Two further groups of experiments were performed. In the first saline was injected in volumes the same as those of adrenalin and failed to alter the white blood count. This proved that the injection *per se* did not excite the animals sufficiently to cause an alteration

¹⁵ West, G. B., *J. Physiol.*, 1947, 106, 426.

Sensitivity of the Lymphopenic Reaction to Adrenalin.*

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Injection of adrenotrophic hormone causes a decrease in the ascorbic acid and cholesterol content of the adrenal cortex (Long and collaborators^{1,2}) and induces a lymphopenia (Dougherty and White³). Since the latter effect is absent in adrenalectomized rats it was interpreted as being due to the increased secretion of adrenocortical hormone resulting from excitation of the adrenal cortex through the adrenotrophic hormone. It was also reported that increased activity of the adrenal cortex accompanied conditions of stress and led to a decrease in ascorbic acid and cholesterol content of the adrenal gland (Long and collaborators), increased excretion of 17-ketosteroids (Pincus,⁴ Hoagland⁵) and lymphopenia (Long and *alii*, Elmadjian and Pincus⁶). Since Vogt⁷ has shown that injection of adrenalin causes an increase in the concentration of adrenocortical hormones in the adrenal vein and since various conditions of stress activate the sympathetico-adrenal system (cf. Gellhorn⁸ for literature) it seemed probable that the alteration in the chemistry of the adrenal cortex as well as the lymphopenia resulted from the secretion of adrenalin which activated the hormones of the adrenal cortex. Such

an interpretation presupposes a great sensitivity of the pituitary-adrenal cortex mechanism to adrenalin. The examples cited by Long indicate that the ascorbic acid content of the adrenal cortex may be lowered significantly by infusion of adrenalin in a dosage of 2 γ 100 g/hr. As far as the effect of the injection of adrenalin on the white blood corpuscles is concerned the data published in the literature suggest that its effect is mainly to produce leucocytosis (Walterhofer,⁹ Naegeli¹⁰). An increase in the number of lymphocytes has been reported by Garrey and Bryan¹¹ whereas a biphasic reaction (leucocytosis followed by leucopenia) was found by Fegler.¹² Our own earlier work showed that conditions such as anoxic and anemic anoxia (Cress, Clare and Gellhorn¹³) and chemically or electrically induced convulsions (Clare, Cress and Gellhorn¹⁴) induce leucocytosis through secretion of adrenalin since this effect was absent in adreno-demedullated animals. In view of the fact that the experiment showing leucocytosis as a result of injection or secretion of adrenalin involved temporal relations quite different from those in which lymphopenia occurred as a result of conditions of stress a reinvestigation of this problem appeared desirable. The experiments reported in this paper show that lymphopenia may result from the injection of minute quantities of adrena-

* Aided by a grant from the Office of Naval Research.

¹ Long, C. N. H., *Fed. Proc.*, 1947, **6**, 461.

² Sayers, G., Sayers, M. A., Liang, T. Y., and Long, C. N. H., *Endocrinol.*, 1946, **38**, 1.

³ Dougherty, T. F., and White, A., *Endocrinol.*, 1944, **35**, 1.

⁴ Pincus, G., Recent progress in hormone research, 1947, **1**, 123.

⁵ Hoagland, H., *J. Comparat. Physiol. Psychol.*, 1947, **40**, 107.

⁶ Elmadjian, F., and Pincus, G., *Endocrinol.*, 1945, **37**, 47.

⁷ Vogt, M., *J. Physiol.*, 1944, **103**, 317.

⁸ Gellhorn, E., *Autonomic Regulations*, New York (Interscience), 1943.

⁹ Walterhofer, G., *Deutsch. Arch. Klin. Med.*, 1921, **135**, 208.

¹⁰ Naegeli, O., *Blutkrankheiten und Blutdiagnostik*, Berlin, 1931.

¹¹ Garrey, W. E., and Bryan, W. R., *Physiol. Rev.*, 1935, **15**, 597.

¹² Fegler, G., *Compt. Rend.*, 1927, **97**, 966.

¹³ Cress, C. H., Clare, F. B., and Gellhorn, E., *Am. J. Physiol.*, 1943, **140**, 299.

¹⁴ Clare, F. B., Cress, C. H., and Gellhorn, E., *Ann. Intern. Med.*, 1944, **21**, 653.

TABLE II.
Effect of Adrenocortical Extract on Action of Adrenalin (0.2 γ /100 g) on Lymphocyte Count of Normal Rats.

No. of rats	Adrenocortical extract	Lymphocytes in %			t	p	Area in mg
		0'	30'	60'			
12	0	100	60	49	8.64	<0.001	172.7
14	3 cc	100	83	81			36.4

cyte count. This confirms in principle the work of Sayers and Sayers and suggests strongly that adrenalin acts primarily on the adrenocortical hormones in the tissues. If their concentration is sufficiently lowered the anterior pituitary is stimulated, more adrenotrophic hormone is secreted which in turn causes an increased secretion of adrenocortical hormones and thereby a replenishment of these hormones in the tissues.

Discussion. Intraperitoneal injection of adrenalin in doses varying from 0.05 to 1 γ /100 g causes in rats a marked lymphopenia while the number of neutrophils increases. However the effect on the lymphocytes is so great that the decrease in the total number of white blood cells is a reliable indicator of the action of adrenalin. This reaction is similar to that observed by Dougherty and White on injection of adrenotrophic hormone. The absence of this effect in adrenalectomized rats is not due to a general deficiency in adrenocortical hormones since the injection of adrenalin fails to alter the white blood count even in the adrenalectomized rats maintained with ample doses of adrenocortical extracts. The data suggest that adrenalin alters the white blood cell count through increased secretion of the adrenocortical hormones. The method is recommended for the assay of adrenalin since even 0.02 γ of adrenalin can be detected in small rats.

The primary factor involved in the action of adrenalin on the lymphocytes seems to be the lowering of the concentration of adrenocortical hormones in the tissues. This interpretation, advanced by Sayers and Sayers¹⁶ on the basis of experiments on the ascorbic acid content of the adrenal cortex, was confirmed by our studies on lymphopenia since in both instances pretreatment with adrenocortical

extracts prevented or greatly diminished the action of stress and of injection of adrenalin.

The lowered concentration of these hormones in blood and tissues furnishes the effective stimulus for the anterior pituitary which through the secretion of adrenotrophic hormone makes an increased secretion of adrenocortical hormones possible. This interpretation makes it understandable that adrenalin produces lymphopenia in normal and adrenalectomized animals even after treatment of the latter with adrenocortical extracts.

The observation of Long¹ that adrenalin does not alter the ascorbic acid concentration of the adrenal cortex in hypophysectomized rats even after injection of anterior pituitary extracts or implantation of the hypophysis does not invalidate this interpretation. It rather suggests that the increased secretion of the adrenotrophic hormone which follows depletion of adrenocortical hormones in the tissues is initiated by impulses in the hypothalamus. This interpretation will be tested in the near future.

Summary. Adrenalin injected intraperitoneally causes neutrophilia and a marked lymphopenia in rats in doses varying from 0.5 to 1 γ /100 g. This effect is absent in adrenalectomized animals and it is greatly diminished in normal rats injected with large quantities of adrenocortical extracts. It is suggested in agreement with Sayers and Sayers that adrenalin increases the consumption of adrenocortical hormones in the tissues and that the lowering of their concentration in the blood leads to increased secretion of the adrenotrophic hormone which in turn increases secretion of adrenocortical hormones. The latter induce lymphopenia.

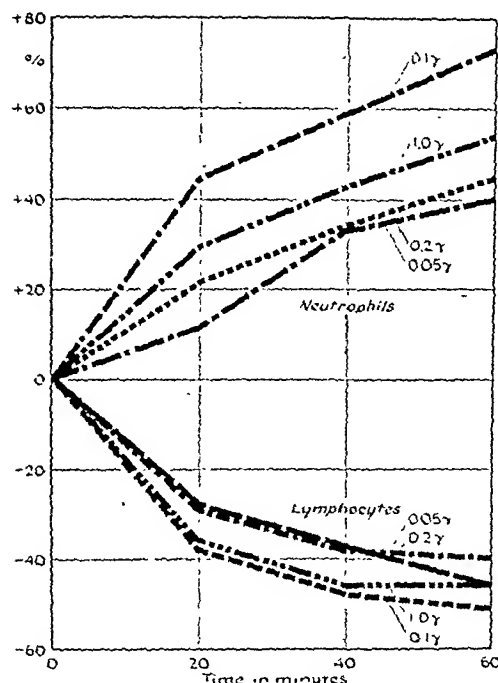


FIG. 1.

The effect of the injection of adrenalin on the percentage changes of neutrophils and lymphocytes. Each graph represents the mean of 10 experiments. Doses of adrenalin of 0.05, 0.1, 0.2, 1 γ /100 g respectively were injected at 0' minutes.

in the white cell count. Also, rats were injected with doses totalling 3 cc of Upjohn's adrenocortical extract[†] in 24 hours, and 2 hours after the last injection (1 cc) the effect of adrenalin was studied. This experiment was performed since Sayers and Sayers¹⁶ had noted that the effect of adrenalin and of various forms of stress on the concentration of ascorbic acid in the adrenal cortex was abolished by pretreatment with adrenocortical hormones. In a control test for this experiment normal rats injected with this extract showed no significant changes in the white blood count between the second and third hour after the last injection of adrenocortical extract. Therefore any alteration in the white count which would occur during this period after the injection of adrenalin could be

causally related to the latter. However, as Fig. 2 shows, adrenalin fails to alter the white cell count of adrenalectomized rats in spite of ample supply of adrenocortical hormones.

This result suggests that the failure of adrenalin to alter the white count in adrenalectomized rats is not due to general metabolic disturbances resulting from the loss of the adrenocortical hormones unless one assumes that the particular adrenal hormone necessary for this response is not present in the extract. However, such an interpretation is incompatible with the following experiment in which the action of adrenalin on the white cell count of normal rats with and without previous treatment with large quantities of adrenocortical extract was studied. Table II shows that the pretreatment of normal rats with adrenocortical extracts greatly reduces the effectiveness of adrenalin on the lympho-

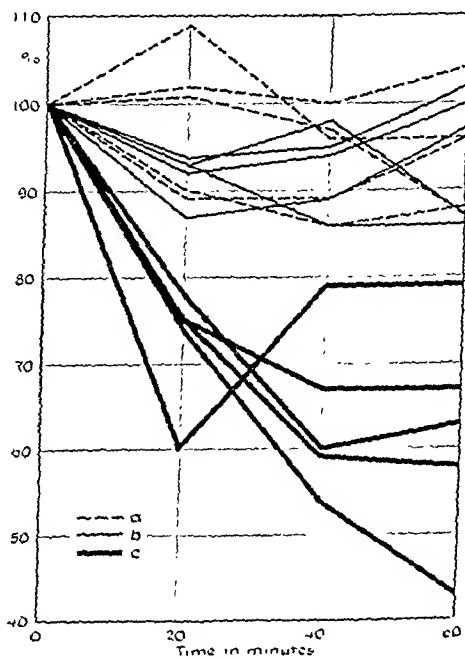


FIG. 2.

The effect of the injection of adrenalin (at 0' min.) on the leucocyte count; a, adrenalectomized rats injected with 3 cc of adrenocortical extract (Upjohn) in 24 hours. Adrenalin (0.2 γ /100 g) administered 2 hours after the last injection of the Upjohn extract; b, adrenalectomized rats injected with 1 γ /100 g of adrenalin; c, normal rats, injected with 0.2 γ /100 g of adrenalin.

[†] Kindly supplied by Drs. Ingle and Hailman.

¹⁶ Sayers, G., and Sayers, M. A., *Endocrinology*, 1947, 40, 265.

activity were also noted at this time. Within a few hours the animal was lying on its side and severe dyspnea was observed. This condition became steadily worse, and no food or water was ingested. This state continued during the second and the third day, the animal becoming progressively weaker until it was unable to rise. Seventy-six hours after withdrawal, when the monkey was removed from its cage to be photographed, excitement and struggling led to further exhaustion and extreme weakness. Respiration was irregular, the animal was cyanotic, and it appeared to be moribund. Emergency measures such as intravenous glucose, injection of caffeine, etc., were attempted in an effort to save the animal, but they were without avail. Postmortem examination indicated an acutely dilated heart but no other significant changes were noted. The animal was generally in a good state of nutrition. Withdrawal signs in the other morphine animal were also severe, but not as striking as those just described.

Since these results are in essential agree-

ment with those previously described by us,² it seems apparent that the normal monkey differs from the dog and from the former human morphine addict in response to this compound. The question must naturally be raised as to whether prolonged poisoning with morphine modifies or conditions the response of the human individual to subsequently administered methadon and/or other compounds and if so whether normal human subjects, previously not addicted to morphine react like the monkey or like the dog. In view of the fact that no primary case of methadon addiction has thus far been described in man, the evidence to date suggests that the response of the normal monkey is similar to that of the non-addict.

Summary. Racemic methadon administered thrice daily in maximal tolerated dose, does not induce a significant degree of physical dependence in the monkey (*Macaca mulatta*) confirming previous experiments involving single daily administration.

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Activity Curves of Crude and Purified Inhibitors and Accelerators of Blood Coagulation.*

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Most crude extracts of tissues possess in a variable proportion, clot inhibiting as well as clot accelerating substances.¹ Ordinarily, the clot accelerators are in dominance, and their action tends to mask the presence of the inhibitors. It seems from the evidence here presented that testing the activity of these extracts or their purified derivatives at different concentrations makes it possible to tell if they consist of such mixtures and, if so,

whether they are *predominantly* clot accelerators or clot inhibitors. Moreover, the relative degree of purity (*i.e.* freedom from the antagonist) of the extracts is reflected by the character of their own activity curves. An extension of this concept has been made to the examination of the coagulant and anticoagulant content of other complex mixtures such as blood, plasma, and plasma fractions.

1. Activity of crude cephalin mixtures. Cephalin was prepared from acetone dried human brain by a method previously described.² *Crude cephalin* refers to the prod-

* Aided by a grant from the U. S. Public Health Service.

¹ Tocantins, L. M., Carroll, R. T., and McBride, T. J., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 110.

² Tocantins, L. M., *Am. J. Physiol.*, 1945, **143**, 67.

Further Observations on Addiction to Methadon in the Monkey.*

J. COCHIN, C. C. GRUHYZT, L. A. WOODS, AND M. H. SEEVERS.

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In a study previously reported, Woods, Wyngaarden, and Seevers¹ found that the monkey (*Macaca mulatta*) did not develop a significant degree of dependence to racemic methadon if the maximum tolerated dose was administered once a day for a period of 4 months. Since these results are at variance with those obtained in the dog which shows marked signs during the first day of withdrawal,² and in former morphine addicts who demonstrate a delayed and atypical abstinence syndrome beginning 5 days after the last dose,³ it becomes essential to know whether a species difference exists in response to this drug; whether previous studies on the monkey were not correctly designed to establish physical dependence because of the long interval between doses (24 hours); or whether the normal monkey responds to the drug like the normal non-addict human (no case of primary addiction to this drug having been reported thus far in this group of individuals). In view of the fact that clinical facilities for studies of addiction may be inadequate to take care of the large number of new analgesics coming to clinical trial, it is important to determine whether animals are of any value for preliminary screening of the addiction potentiality of new compounds.

To obtain further information, racemic methadon was administered to 4 monkeys thrice daily in initial dosage of 2 mg/kg and raised to 7 mg/kg within 66 days, and con-

tinued thereafter on this regimen until the termination of the experiment. Increase in the dosage above 7 mg/kg resulted in excessive depression, loss of appetite and weight, but the animals remained in excellent condition on this dose. The two morphine controls were raised from initial dosage of 5 mg/kg (15 mg/kg daily) to 50 mg/kg (150 mg/kg daily) in 112 days and continued thereafter on this dose.

After 142 days of poisoning, both drugs were withdrawn and observations were made through a one-way vision glass panel, a technical installation which eliminates modification of the animal's behavior by the presence of the observer. Abstinence signs⁴ were absent or minimal after methadon was withdrawn. Very slight pilomotor activity was present in all 4 animals after 30 hours, but disappeared at the end of 72 hours. After 36 hours, the monkeys were somewhat more irritable than during the period of administration but this, we believe, is a manifestation of the elimination of the slight depression produced by the methadon and a return to normal excitability rather than a true state of hyperexcitability. No anorexia was noted. One animal showed moderate inflammation of the eyelids and cornea on the fourth day of withdrawal and two had slight diarrhea on the fourth day. Otherwise no significant changes were observed.

Very marked signs were noted in both animals withdrawn from morphine. One of these animals showed the most dramatic and severe abstinence signs ever observed by the senior author. This animal showed lacrimation, inflammation of the eyelids and cornea 20 hours after withdrawal. Shivering, facial perspiration, chattering, intention tremor, muscular rigidity, twitching, and pilomotor

* Supported by a grant-in-aid from the United States Public Health Service.

¹ Woods, L. A., Wyngaarden, J. B., and Seevers, M. H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 113.

† Through the courtesy of Dr. C. A. Bratton of Parke Davis & Co.

² Wikler, A., and Frank, K., *Fed. Proc.*, 1947, **6**, 384.

³ Isbell, H., Wikler, A., Eddy, N. B., Wilson, J. L., and Moran, C. F., *J.A.M.A.*, 1947, **135**, 888.

⁴ Seevers, M. H., *J. Pharm. Exp. Therap.*, 1936, **50**, 147.

and anticoagulant fractions of the crude extracts have antagonistic actions, and moreover, their curves of activity (Curves B and C) no longer have the biphasic character of the crude preparation (Curve A, Chart 1). Freed of the inhibitor, the purified cephalin at 1.4 percent concentration is a more potent coagulant than the crude cephalin. The monophasic curves seem to represent the activity of preparations fairly free of their respective antagonists.

3. Activity of artificial coagulant-anticoagulant mixtures. These were prepared by mixing different proportions of solutions of the purified coagulant and anticoagulant fractions of the brain. The curves of activity for these mixtures (D,E,F, Chart 3) follow a course analogous to that of crude cephalin preparations. The mixtures with more coagulant (Curve F) exhibit the biphasic behavior at the higher concentrations, while those with more anticoagulant (Curve D) display it at lower concentrations.

The foregoing led us to test the activities of various fluids such as blood, plasma and cer-

ACTIVITY OF THREE MIXTURES
OF PURIFIED COAGULANT
AND ANTICOAGULANT
FRACTIONS FROM BRAIN TISSUE

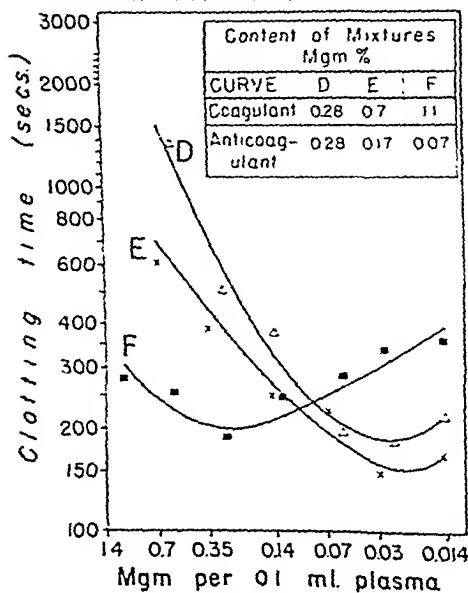


CHART 3.

EFFECT OF DILUTION (0.85% NaCl)
ON THE RATE OF COAGULATION
OF VENOUS BLOOD
OF NORMAL ADULTS

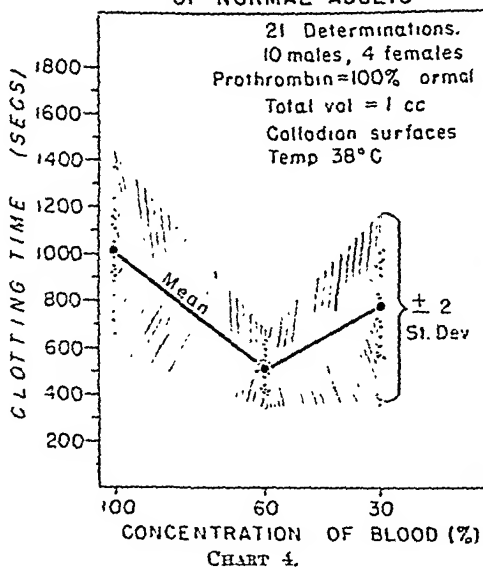


CHART 4.

tain plasma fractions separated by the Cohn method.⁵

Activity curves of blood and plasma. Blood was collected with special precautions³ and placed into 3 collodion coated tubes. One ml was placed in the first tube; the amount of blood in each of the other 2 tubes was so adjusted that in the second tube the blood was diluted to 60% of its volume (0.6 ml blood, 0.4 ml 0.85% NaCl) and in the third tube to 30% (0.3 ml blood, 0.7 ml 0.85% NaCl). All three tubes were gently tilted 2 or 3 times and kept at 38°C. As shown in Chart 4, dilution of the blood to 60% of its volume accelerates its coagulation, and even when diluted to 30%, the blood still clots faster than when undiluted. Blood from hemophiliacs is even more strikingly affected by dilution (Chart 5).

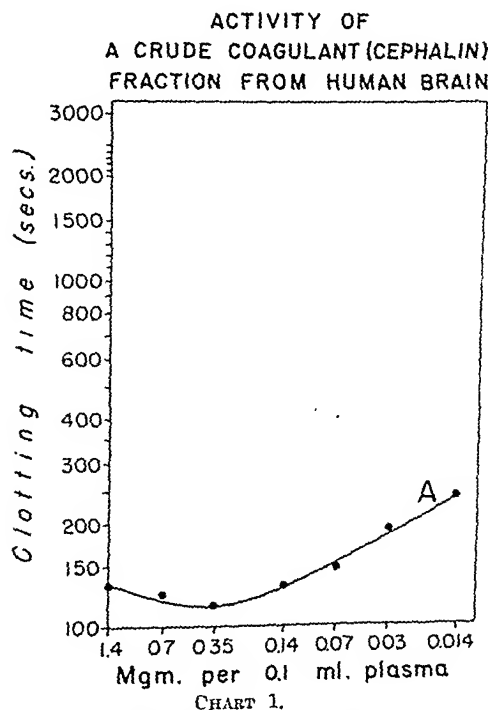
Hypercoagulable blood, as observed after a severe hemorrhage, is little affected by contacting surfaces of different types⁶ and clots nearly as rapidly in glass as in collodion tubes. Dilution of such blood prolongs its rate of coagulation from the start (Chart 6), an indi-

⁵ Cohn, E. J., *Blood*, 1948, 3, 471.

⁶ Tocantins, L. M., *Blood*, 1946, 1, 156.

uct of only one precipitation of the ether-soluble lipid with cold absolute ethanol. This precipitate was washed once with acetone, the acetone removed and the waxy material well homogenized in a 1.4 g% solution in 0.85% NaCl, the pH being adjusted to 7.2 - 7.4. The activity of the preparation was then tested against citrated normal human plasma, collected and preserved with special precautions³ and measured with collodion coated pipettes.² The clotting time of such plasma in collodion coated 13 mm wide tubes, at 38° (0.1 ml plasma, 0.1 ml 0.85% NaCl, 0.1 ml 0.02 M CaCl_2) ranges between 500 and 700 seconds.

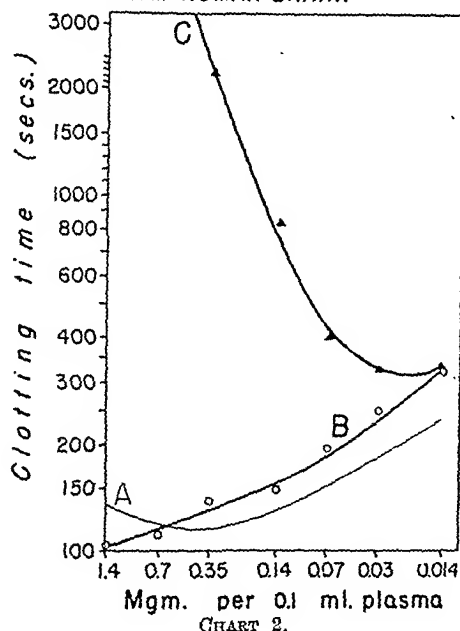
Testing the activity of this crude cephalin at decreasing concentrations yields a curve with a biphasic course (Chart 1). The greatest clot accelerating effect was obtained at a concentration of the lipid extract in the plas-



Cephalin obtained from a single precipitation with absolute ethanol from an ether extract of human brain. In this and subsequent similar charts, the abscissa refers to total mg of the lipid coagulant or anticoagulant (or a mixture of both) added to 0.1 ml of recalcified citrated plasma.

³ Tocantins, L. M., *Am. J. Physiol.*, 1943, 139, 265.

ACTIVITY OF PURIFIED CEPHALIN (B), AND ANTICOAGULANT (ANTI-THROMBOPLASTIN) (C) FRACTIONS FROM HUMAN BRAIN.



ma of 0.35 g%, which corresponds well with the optimum range of concentration (0.06 - 1.0%) for the activity of crude cephalin observed by Hanzlik and Weidenthal.⁴ The crude cephalin at first gains clot accelerating power on dilution, then reverses itself and progressively becomes less potent. Such biphasic activity curves seem to be an expression of the coexistence of coagulants and their antagonists.

2. Activity of purified extracts. The crude cephalin obtained by a single precipitation of the ether-soluble lipid was then purified by repeated precipitations with cold absolute ethanol. "Purified cephalin" is used to designate the ethanol insoluble lipid obtained after six successive precipitations. The mother liquors treated as described in method 1, elsewhere¹ yielded a clot-delaying lipid, here designated as "purified anticoagulant" (or anti-thromboplastin).

As shown in Chart 2, the purified coagulant

⁴ Hanzlik, P. J., and Weidenthal, C. M., *J. Phar. and Exp. Ther.*, 1919, 14, 157.

EFFECT OF SOLUTIONS OF VARIOUS PLASMA FRACTIONS (S & D) AT DIFFERENT pH, ON THE COAGULATION OF NORMAL PLASMA

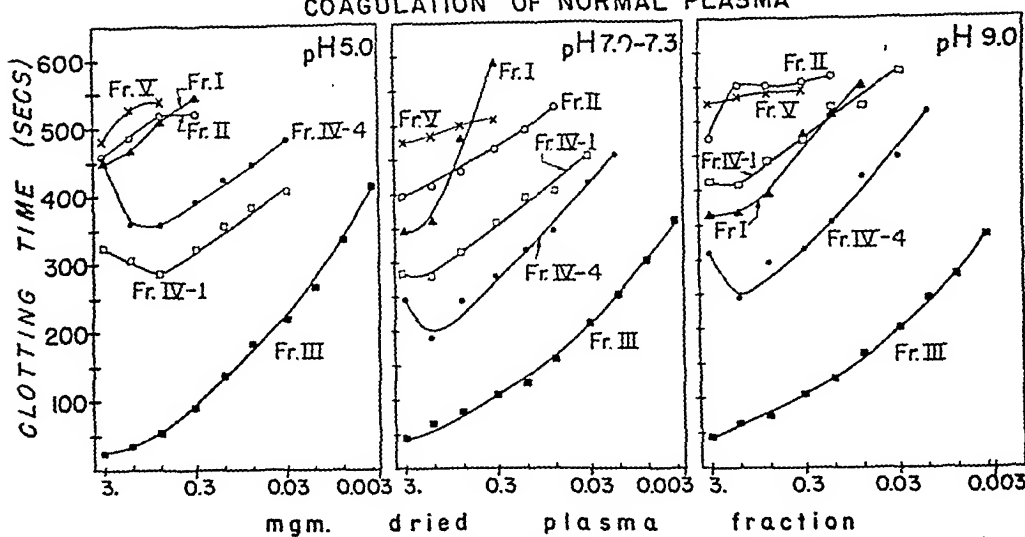


CHART 7.

The pH of the solutions was adjusted with dilute NaOH or HCl. A 3% solution of each fraction in H₂O was prepared; dilutions were made with 0.85% NaCl. 0.1 ml fraction, 0.1 ml normal citrated plasma, 0.1 ml CaCl₂ (optimum molar concentration for each fraction/plasma mixture.) Fraction I usually contains the largest amount of citrate.

especially the latter, were of the biphasic type. Testing the activity of the fractions in an acid solution (pH 5.) seemed to accentuate the biphasic behavior of fractions IV-1 and IV-4. Even at pH 9, however, the trend of the curves was not significantly altered (Chart 7). The course of the curves suggested that these two fractions contained anticoagulant factors. A good portion of the lipoprotein complexes of the plasma is in fraction IV,⁸ and it has also yielded the highest content of the lipid antithromboplastin.⁹

Discussion. These observations seem to make it desirable that the activity of purported clot-accelerating and inhibiting substances be tested over a wide range of concentrations, using stable plasma as a substrate, held in surfaces which do not themselves accelerate coagulation. Only in this

manner may it be possible to detect both coagulant and anticoagulant activity, since excess of one may mask the action of the other, when the material is tested at a single concentration. The most striking example of this behavior is found in fraction IV-4 from which an active anticoagulant may be separated,⁹ yet the intact fraction is a clot accelerator.

The slope of the activity curve of the purified anticoagulant is much steeper than that of the purified coagulant. Within the range of 0.35 - 0.07 mg the relation between concentration of the material and clotting time is linear (Chart 2). The anticoagulant gains (or loses) activity in this range, at a considerably more rapid rate than the coagulant. The activity curves of the artificial mixtures (Chart 3) compromise between the steep slope of the purified inhibitor and the gradual straight rise of the purified coagulant.

The biphasic course of the activity curves of normal blood and plasma seem to indicate that these fluids themselves are complex anti-coagulant-coagulant mixtures. The fact that diluted blood, though containing less pro-

⁸ Mulford, D. J., *Ann. Rev. Physiol.*, 1947, 9, 327.

⁹ Carroll, R. T., and Tocantins, L. M., Separation of a Lipid Antithromboplastin from Blood, Plasma and Plasma Fractions; presented at the International Congress of Hematology, Buffalo, August 23, 1948.

EFFECT OF DILUTION (0.85% NaCl)
ON THE RATE OF COAGULATION
OF VENOUS BLOOD
OF THREE HEMOPHILIC MEN.

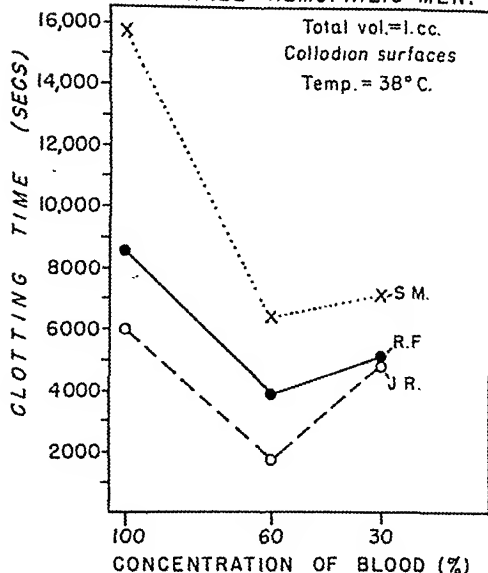


CHART 5.

Dilutions carried out as explained in text. Prothrombin concentration of undiluted blood (1-stage method) 100% of normal.

EFFECT OF DILUTION (0.85% NaCl) ON THE
RATE OF COAGULATION
OF THREE TYPES OF BLOOD AND PLASMA

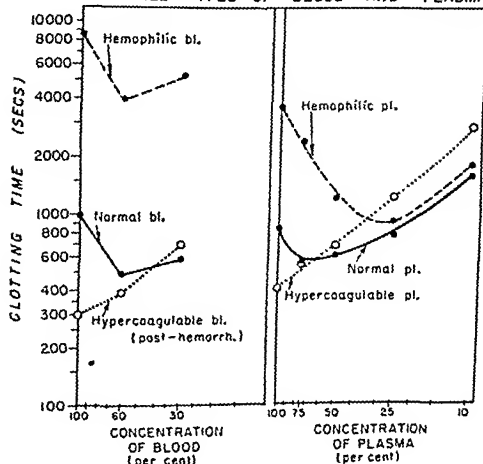


CHART 6.

Effect of dilution on the rate of coagulation of hemophilic, normal, and post-hemorrhagic blood and plasma. Blood diluted as described in text. Plasma clotting mixtures: 0.1 ml plasma, 0.1 ml 0.85% NaCl, 0.1 ml CaCl_2 (molar concentration adjusted to dilution of plasma); collodion coated tubes at 38° C.

cation that coagulants are predominant, the inhibitors having been reduced either by blood dilution *in vivo* during the hemorrhage, or offset by entrance of coagulants (thromboplastin) into the circulation.

The effect of dilution on hypocoagulable (hemophilic), normal and hypercoagulable (post hemorrhagic) plasmas is similar to that on whole blood (Chart 6). Hypercoagulable plasma which when undiluted clots many times faster than hemophilic plasma, actually clots slower than the latter, when both are diluted to below 20% of their original concentration. If the curves on Chart 6 are compared with those of the mixtures of purified coagulant and anticoagulant (Chart 3), it is apparent that the hemophilic plasma behaves like a predominantly anticoagulant mixture (Curve E, Chart 3) while the post hemorrhagic plasma is more like a predominantly coagulant solution (Curve B, Chart 2).

The clot-accelerating effect of dilution is best demonstrated in paraffin or collodion.² In glass the principal effect of dilution is to prolong the time,⁷ for glass itself accelerates coagulation and dilution of the blood will only reduce that effect. Even in glass, however, the clotting time of hemophilic plasma is substantially reduced by dilution,² though the changes are not as impressive as in collodion or plastic tubes.

5. Activity Curves of Plasma Fractions. The dry plasma fractions were obtained from Dr. Cohn's laboratory and the biological laboratories of Sharp and Dohme. Three percent solutions were made in 0.85% NaCl, and the pH adjusted to between 7.0 and 7.3. The fractions were tested on recalcified citrated normal human plasma, as described before.

With the exception of fraction V all fractions had clot-accelerating activity at concentrations between 0.7 and 3%. Fraction III was the most potent due perhaps to its prothrombin and thrombin content.⁵ Fraction V was apparently inert; it did not seem to have either coagulant or anticoagulant action. The curves of activity for fractions IV-1 and IV-4,

⁷ Copley, A. L., and Houlihan, R. B., *Science*, 1944, 100, 505.

TABLE I.

Simultaneous Urinary Pregnanediol Determinations by the Venning Method and the Free Pregnanediol Method in Normal Pregnant Patients and in Pregnant Patients Who Were Taking Diethylstilbestrol.

No.	Free preg., mg/24 hr	Administered diethylstilbestrol, mg	NaPG* calculated as preg., mg/24 hr
1	11.93	0	7.91
2	19.72	0	15.54
3	19.68	0	18.00
4	28.99	0	28.75
5	35.40	0	33.80
6	48.80	0	48.70
7	11.53	25	13.98
8	41.90	50	60.44
9	11.64	50	35.26
10	36.85	100	69.15
11	28.73	115	75.42
12	20.77	125	60.10
13	31.05	125	83.55
14	51.46	125	96.13

* These are uncorrected results. No attempt has been made to account for losses during extraction.

method for the determination of urinary pregnanediol measures the glucuronide titer of the urine. Thus, other glucuronides than pregnanediol are included in the results obtained. The method that has been in use in our laboratory determines the amount of free pregnanediol after acid hydrolysis.³ The following experiments were carried out to compare the results obtained by these two methods in normal pregnant women who were receiving no medication and in those to whom diethylstilbestrol in varying amounts was administered.

Methods and results. A series of 24 hour urine collections from normal women during various periods of gestation were analyzed simultaneously by the Venning method and by the method for free pregnanediol used in our laboratory. There was an extremely close correlation in the results obtained by the two procedures (Table I). The figures tabulated are uncorrected since it seemed to us that some loss of pregnanediol would occur in both methods.

Simultaneous pregnanediol determinations by both methods were made in a second group

of pregnant patients to whom varying amounts (25 to 125 mg) of diethylstilbestrol were administered daily. The Venning method yielded substantially higher values than the method used in our laboratory. Furthermore, the increase obtained by the Venning method over that obtained by the free pregnanediol procedure was roughly in proportion to the amount of diethylstilbestrol administered to the patient.

A small group of patients in various stages of pregnancy under observation in the hospital was used for the following experiment. The urine was collected for several 24 hour periods and urinary pregnanediol levels were determined by both methods. The patients then received a single oral dose of 200 mg of diethylstilbestrol and urine collections continued. These, too, were analyzed for pregnanediol. The results of the determinations prior to the administration of diethylstilbestrol were comparable in both procedures. However, there was a marked increase in the amount obtained by the Venning method following the administration of the estrogen. (Fig. 1) Furthermore, in most cases this increase occurred during the 24 hour period following the ingestion of the diethylstilbestrol but in one patient there was a short delay so that the increased amount did not appear until

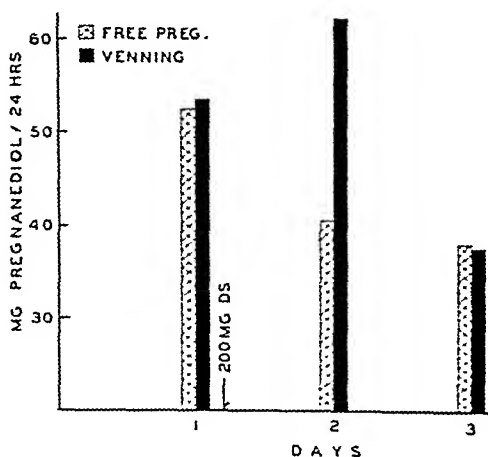


Fig. 1.

Simultaneous urinary pregnanediol determinations by the Venning method and the free pregnanediol method in a normal pregnant patient before and after the administration of diethylstilbestrol.

⁷ Smith, A. E. W., and Williams, P. C., *Biochem. J.*, 1948, 42, 253.

⁸ Venning, E. H., *J. Biol. Chem.*, 1937, 119, 473.

thrombin, platelets, ac-globulin and fibrinogen clots faster than intact blood, is an indication of the effectiveness of inhibitors in blocking or offsetting changes in these coagulation factors. Moreover, since the coagulation of hemophilic blood or plasma is greatly shortened by dilution, it is difficult to understand how a deficiency of a plasma constituent can be responsible for the defect in this disorder. Dilution would naturally tend to accentuate the deficiency and thereby further delay coagulation.

Summary. Crude and purified cephalin

and antithromboplastin extracted from brain tissue yield characteristic activity curves when tested at different concentrations, on stable citrated normal human plasma. Moderate dilution accelerates the coagulation of normal and hemophilic blood and plasma and delays that of posthemorrhagic blood and plasma. Excepting fraction V, all Cohn's plasma fractions have coagulant action; only fractions IV-1 and IV-4 display a biphasic curve of activity, an indication that they are mixtures of coagulants and anticoagulants.

16745

Does Administration of Diethylstilbestrol to Pregnant Women Result in Increased Output of Urinary Pregnanediol?*

M. EDWARD DAVIS AND NICHOLAS W. FUGO.

From the Department of Obstetrics and Gynecology, and of Pharmacology, The University of Chicago and The Chicago Lying-in Hospital.

In 1946 there appeared in the literature the interesting observation that the oral administration of diethylstilbestrol to a pregnant diabetic woman resulted in an increased excretion of urinary pregnanediol as measured by the Venning method.¹ This finding was interpreted by the authors as an index of increased production of steroids by the placenta brought about by estrogenic stimulation. They suggested the oral administration of diethylstilbestrol in the prevention and treatment of the accidents of late pregnancy such as the toxemias, premature fetal death and diabetic complications associated with gestation.

In an earlier report² we were unable to demonstrate increased pregnanediol excretion in patients receiving large amounts of diethylstilbestrol early in pregnancy. During the past 2 years we have studied a large group of

patients who presented a variety of pregnancy complications in early and late gestation. Large amounts of diethylstilbestrol (5 to 200 mg daily) were administered to these women and bi- and tri-weekly urinary pregnanediol determinations were made by a method described by us in a previous communication.³ No obvious increase in free pregnanediol was apparent in any of our studies. The results of these observations are in the process of publication.

The apparent discrepancy in the results obtained from the administration of diethylstilbestrol to pregnant women by the Smiths and in our own laboratory led us to seek an explanation. It has been reported in numerous publications⁴⁻⁷ that in the metabolism of diethylstilbestrol it is conjugated and ultimately excreted as a glucuronide. The Venning

* This work has been done under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

¹ Smith, O. W., Smith, G. V. S., and Hurwitz, D., *Am. J. Obst. and Gynec.*, 1946, **51**, 411.

² Davis, M. E., and Fugo, N. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 283.

³ Davis, M. E., and Fugo, N. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 39.

⁴ Zondek, B., and Sulman, F., *Nature*, 1939, **144**, 596.

⁵ Stroud, S. W., *J. Endocrinology*, 1939, **1**, 201.

⁶ Mazur, A., and Shorr, E., *J. Biol. Chem.*, 1942, **144**, 283.

the female during the estrus cycle. (2) The male sex hormone might have exerted an antagonistic effect on the carcinogenic action of the estrogenic hormone.⁷⁻⁸

The present experiments were carried out in order to obtain further information concerning the role of the age factor in estrogen-induced mammary cancers.

Material and methods. One hundred and twenty-three male mice of the closely inbred strain C3H, raised in our laboratory, and kept on a standard diet of Purina Laboratory Chow and water were used. Sixty-five animals were castrated at the age of 3 to 4 weeks. Twenty-three castrates (Group I) and 26 animals with intact testicles (Group II) received subcutaneous injections of 0.03 mg (200 Rat Units) of alpha estradiol benzoate† in sesame oil once a week for 5 months starting at the age of one month. The remaining 42 castrates (Group III) and 32 mice with intact testicles (Group IV) were injected with the same amount of hormone for the same length of time, but the treatment was begun at the age of 4 months. The animals were inspected at weekly intervals for the appearance of tumors and leukemia. Dead animals were examined for gross lesions. Tumor-bearing mice or animals which looked sick were killed. The tumor and 3 or 4 mammary glands, pieces of internal organs and some bones were removed and saved for histological studies. These findings will be reported at a later date.

Observations. The results are summarized in Table I. The first breast tumors were noted at the age of 7 months, and, therefore, mice living to this age and beyond are listed as animals "reaching the tumor age". These animals were distributed, as follows:

Group I: Castrates receiving the hormone from 4 weeks of age on: 18.

⁵ Lacassagne, A., and Raynaud, A., *Compt. Rend. Soc. Biol.*, 1939, 131, 186.

⁶ Nathanson, I. T., and Andervont, H. B., *Proc. Soc. Exp. Biol. and Med.*, 1939, 40, 421.

⁷ Gardner, W. L., *Cancer Res.*, 1946, 6, 493.

⁸ Jones, E. E., *Cancer Res.*, 1941, 1, 787.

† We are indebted to the Schering Corporation for the generous supply of Progynon-B.

TABLE I.

Experimental group	Total No. of animals	No. of animals reaching tumor age	Age at death of animals reaching tumor age		Animals with breast cancers		
			Range	Mean (mo.)	No.	Age at appearance of tumors	
						Range	Mean (mo.)
I. Male castrates receiving hormone at 4 wk	23	18	7-18	12.9	8	7-15	9.7
II. Males with intact testicles receiving hormone at 4 wk	26	19	7-17	12.8	3	8-14	10.7
III. Male castrates receiving hormone at 4 mo.	42	40	7-19	13.0	12	8-19	12.8
IV. Males with intact testicles receiving hormone at 4 mo.	32	25	7-20	10.8	1	--	17.0

the second 24 hour urine collection. This patient had serious kidney damage which may have accounted for the delay in excretion. No attempt was made to determine the amount of diethylstilbestrol excreted in the feces.

Finally, the material obtained from the Venning procedures was assayed qualitatively for estrogenic activity using the castrated female guinea pig as the test animal. Opening of the vaginal canal by dissolution of the vaginal membrane was taken as the index of estrogenic activity. It was found that only those samples which showed an increased pregnanediol titer with the Venning method over the free pregnanediol method exhibited estrogenic potency during the period of observation.

Summary and conclusions. When diethylstilbestrol is administered to a woman during pregnancy, much of this material appears in the urine as a glucuronide. In the Venning method for the assay of urinary pregnanediol, all of the glucuronides are included in the final determination. Thus diethylstilbestrol

glucuronide as well as pregnanediol glucuronide appears in the result obtained. The apparent rise in the urinary pregnanediol values obtained by the Venning method may be due to the ingested diethylstilbestrol which is conjugated and eliminated in the urine. The figures do not indicate an increased production of progesterone and resultant increased output of urinary pregnanediol.

Increasing amounts of diethylstilbestrol are being used in the treatment of pregnancy complications. In most instances the theory behind this therapeutic measure is that this estrogen stimulates steroid production. If urinary pregnanediol is to be used as a measure of increased steroid metabolism the value of diethylstilbestrol is open to question. It is possible that diethylstilbestrol exerts a favorable influence on placental circulation or on early placental development. However, there is no evidence that it results in an increased production of progesterone if urinary pregnanediol is to be regarded as an index of progesterone metabolism.

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Age Factor in Estrogen-Induced Breast Cancers of Mice.*

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In male mice of strains C3H and D, the age at the beginning of the treatment was one of the factors which determined the susceptibility of these animals to estrogen-induced breast cancers¹ and leukemias.²⁻⁴ The breast

cancer rate was higher in mice injected with estrogenic hormone before or about the onset of sexual maturity than in those receiving the hormone from the age of 4 to 6 months on. In female mice similarly treated, this age effect was much less conspicuous or lacking. According to Loeb,^{1b} these findings suggest the following interpretation: (1) In younger mice, the breast tissue itself might be more susceptible to growth stimulation than in older animals. If this is the case, however, the lack of an age effect in females would be difficult to explain. Still, such an age factor might be present in female mice also, but it might be obscured or neutralized by the periodic stimulation of the mammary gland by the intrinsic estrogenic hormone produced in

* This investigation was supported by a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

1 (a) Loeb, L., *Harvey Lectures*, 1941, **36**, 228; (b) Loeb, L., *Biol. Symposia*, 1945, **11**, 197; (c) Loeb, L., Suntzeff, V., Burns, E. L., and Schenken, I. R., *Arch. Pathol.*, 1944, **38**, 52.

2 Murphy, J. B., *Cancer Res.*, 1944, **4**, 622.

3 Silberberg, M., and Silberberg, R., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 347.

4 Law, L. N., *J. Nat. Inst. Canc.*, 1947, **8**, 157.

than in the older ones (Group IV), the mean tumor age being in Group II 10.7 months, and in Group IV 17 months. Three months of this interval can be accounted for by the 3 months' delay of the hormonal treatment in the older group. However, the additional 3.3 months' difference has to be attributed to conditions of the experiment. Although the absolute number of tumors observed in these two age groups is small, the increase in the latent periods is in agreement with the decrease in the tumor incidence. It is, therefore, not unlikely that both are governed by

similar factors.

Summary. Male mice of strain C3H castrated at the age of 3 to 4 weeks and injected with alpha estradiol benzoate immediately after castration develop a larger number of breast cancers than males castrated at the same age but injected from the age of 4 months on. Thus, an age factor operates in the susceptibility to estrogen-induced mammary cancers in male mice. This age factor is independent of the testicle but acts synergistically with it.

16747

Effect of Insulin and Glucose Upon Survival Time of Eviscerated Rats.

DWIGHT J. INGLE AND JAMES E. NEZAMIS.

From the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

The data of these experiments show that the administration of glucose with insulin to the eviscerated rat permits longer survival times than the administration of glucose alone.

Methods. Male rats of the Sprague-Dawley strain were fed Friskies Dog Cubes. At a weight of 185 to 205 g, the inferior vena cava was ligated between the liver and the kidneys in order to cause the development of a collateral circulation. Asepsis was preserved in this operation. When the animals reached a weight of 250 ± 2 g they were anesthetized (intraperitoneal injection of 18 mg of cyclopentenylallyl-barbituric acid sodium) and eviscerated by the method of Ingle and Griffith.¹ Hemostasis was attained by applying a gelatin sponge (Gelfoam, Upjohn) with thrombin to the stumps of the oesophagus, colon, ligated vessels and between the muscle and the skin when the incisions were closed. The animals were not fasted prior to operation. Intravenous infusions of solutions of glucose (C.P. Dextrose, Merck) in 0.9% saline with and without added insulin (Crystalline zinc in-

sulin, Lilly) were made by continuous injection machines which delivered fluid into the saphenous vein of the right hind leg at the rate of 20 cc per 24 hours. The glucose loads used in this experiment represent amounts which sustain the average level of blood glucose at approximately normal values during a period of 24 hours. Temperature was constant at $26.5 \pm 0.5^\circ\text{C}$.

The time at which the heart of the rat stopped beating was determined by use of a heart-beat amplifier (Model A, Upjohn) which was designed to amplify the D.C. potential generated by the heart beat to actuate a 6-point recording mechanism (Leeds and Northrop Micromax S.) This apparatus gives a permanent visual record of an all-or-none response of the amplifier to the beating heart for 6 animals simultaneously.

In Experiment 1, 21 eviscerated rats were given a glucose load of 4 mg per 100 g of rat per hour (4/100/h). The average survival was 1748 minutes \pm 113 (standard deviation of the average). Glucose loads of 4/100/h without insulin and 40/100/h with insulin are each able to sustain approximately normal levels of blood glucose during 24 hours

¹ Ingle, D. J., and Griffith, J. Q., Chapter 16, *The rat in laboratory investigation*. J. B. Lippincott Co., Philadelphia, 1942.

Group II: Controls receiving the hormone from 4 weeks of age on: 19.

Group III: Castrates receiving the hormone from 4 months of age: 40.

Group IV: Controls receiving the hormone from 4 months of age on: 25.

In males with intact testicles injected with alpha estradiol benzoate for 5 months from the age of one month on (Group II), the cancer rate was 15.8%; the tumors appeared at a mean age of 10.7 months. In males with intact testicles treated similarly but receiving the hormone from the age of 4 months on (Group IV), the cancer incidence was 4%; the single tumor observed developed at the age of 17 months. Thus, in the younger control group, the estrogen-induced neoplasms were almost 4 times more numerous, and they appeared 6.3 months earlier than in the older control group. These results are essentially in agreement with the findings obtained by Loeb and his co-workers.¹⁰

In males castrated at the age of 3 to 4 weeks and injected immediately with alpha estradiol benzoate for 5 months (Group I), the incidence of tumors was 44.4%; the neoplasms appeared at a mean age of 9.7 months. Orchidectomy thus raised the tumor rate in this age group about threefold and accelerated the appearance of the cancers by one month. Of males castrated at the age of 3 to 4 weeks but receiving the estrogenic hormone from the age of 4 months on (Group III), 30% developed mammary cancers as compared with 44.4% in the group injected at an earlier period of life, and the tumors were noted at a mean age of 12.8 months. Thus, in the older age group, castration increased the incidence of estrogen-induced breast cancers $7\frac{1}{2}$ times and advanced the time of their appearance by about 4.2 months over that seen in mice with intact testicles.

Discussion. Orchidectomy performed before the onset of sexual maturity raised the incidence of estrogen-induced breast cancers. This increase was observed not only if the hormonal treatment was begun immediately after castration⁹ but also, if the injections were

started as late as 3 months after removal of the testicles. However, the results obtained in these 2 age groups differed in degree: Castrates injected from the age of one month on, showed a cancer rate about 50% higher than those in which the hormonal treatment was begun at the age of 4 months (44.4% in castrates of Group I as compared with 30% in castrates of Group III). These two groups of castrates thus showed variations in the incidence of estrogen-induced breast cancers similar to those occurring in the corresponding mice with intact testicles. However, whereas in the latter animals the tumor rate of the two age groups differed as much as 300%, the difference in injected castrates of the two age groups amounted to about 50%. This latter difference in the cancer rate of both age groups thus occurred in the absence of the testicles. It can, therefore, not be attributed to any inhibiting effect of the sex glands but it must be caused by certain extra-testicular factors. It would be premature to define more precisely the nature of this age factor. It is presumably located in the mammary gland itself and constitutes a loss of responsiveness to estrogenic stimulation with advancing age, an interpretation which would be in agreement with the suggestion of Loeb.¹¹

A decrease of susceptibility may manifest itself not only in a lowered cancer incidence but also in a prolongation of the latent period of tumor development. In our present experiments, the latent period was apparently not influenced by the age of the castrates at the beginning of the hormonal treatment: In the younger castrates (Group I), the estrogen-induced breast cancers appeared 3.1 months earlier than in the older castrates (Group III), the mean tumor age being 9.7 months in the younger and 12.8 months in the older group. This difference in time corresponds to the 3 months' delay of the administration of the estradiol. This result may be correlated to the comparatively small difference in the tumor incidence of the two groups. On the other hand, in younger animals with intact testicles (Group II), estrogen-induced breast cancers appeared 6.3 months earlier

⁹ Miller, E. W., and Pybus, F. C., *J. Pathol. and Bact.*, 1942, 54, 155.

the action of diethyldithiocarbamate upon the oxygen uptake of active and blocked cells of the embryo of the grasshopper, *Melanoplus differentialis*. This compound is of especial interest since it has been extensively employed in methods dealing with the quantitative determination of copper. Loose physico-chemical compounds of copper are readily formed with it and this property, along with that of its being a carbamate, make it of especial interest for problems of cellular physiology.

Material and methods. The embryo of the grasshopper has proven to be extremely favorable biological material for investigating cellular reactions since it can be obtained both in a growing or mitotically active state as well as in a resting or blocked condition in which no mitotic activity is present.⁴ Embryos were dissected from eggs in sterile phosphate buffered Ringer solution (pH 6.8) and treated as previously indicated.⁵ All solutions of sodium diethyldithiocarbamate were made up in Ringer solution and final concentrations were calculated from the amounts added to the respiration flasks. Standard Warburg techniques with flasks of 5 cc capacity were employed at 25°C. One hundred embryos were used in each flask and 12 to 18 manometers employed for each concentration of reagent.

Results. All remarks concerning the action of diethyldithiocarbamate should be prefaced with the fact that no significant differences in the reactions of mitotically active and blocked cells to this reagent have been found. Data presented, therefore, apply equally well to both physiological states of the cells.

The qualitative nature of the response of the oxygen intake of the embryonic cells to the reagent is strikingly similar for all concentrations employed. However, differences in degree of response, due to increased concentrations of the reagent, occur. Low concentrations produce only stimulation. In general (Fig. 1), an initial inhibitory period followed by a marked increase in oxygen consumption is noted for all high concentrations

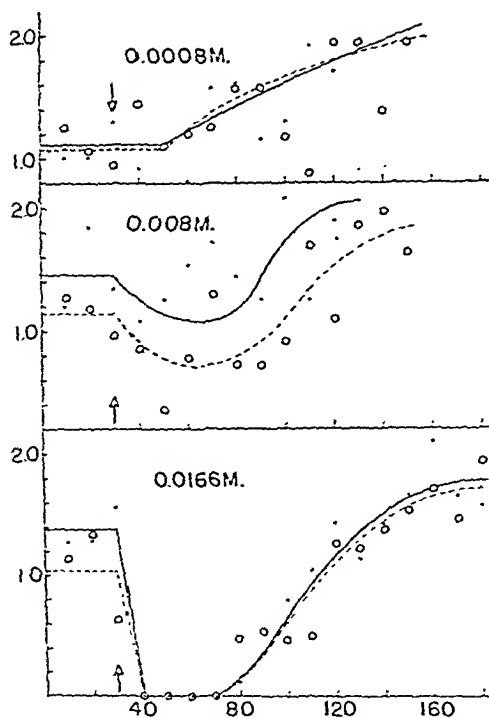


FIG. 1.

Shows effect of concentrations of diethyldithiocarbamate on the oxygen consumption of active and blocked cells of the embryo. Solid circles, active cells; open circles, blocked cells. Abscissa, time in minutes; ordinates, mm³ O₂ per 100 embryos per 10 minutes. Arrow indicates time of addition of drug. Concentrations in molarity indicated.

of the reagent. The length and degree of the inhibition and stimulation seem directly related to the concentration of the drug.

Embryos exposed to high concentrations for periods of 30 to 60 minutes and then washed and resuspended in Ringer solution, show almost complete recovery as indicated graphically in Fig. 2. As a matter of fact, the response of the embryo left exposed constantly to the reagent for periods comparable to those for washed embryos in Ringer solution, as in the above recovery experiments, show much higher rates of oxygen intake. Apparently constant exposure to the reagent is essential for the types of response normally shown by the embryos.

Since diethyldithiocarbamate is definitely known to unite with copper it becomes of some

⁴ Slifer, E. H., *J. Morph. and Physiol.*, 1931, 51, 613.

⁵ Bodine, J. H., and Fitzgerald, L. R., *Physiol. Zool.*, 1948, 21, 303.

and longer. Twenty-two similar rats were given a glucose load of 40/100/h with 4 units of insulin per 24 hours per rat. The average survival was 2017 ± 77 minutes. The standard deviation of the difference (269) between the averages was 137 giving a ratio of 1.96 between the difference and its standard deviation, thereby indicating a statistical probability of 95 chances in 100 that the average survival of insulin treated rats is greater than the average survival of rats not treated with insulin.

In Experiment 2, 20 eviscerated rats were given a glucose load of 6/100/h without insulin. The average survival was 1828 ± 114 minutes. Twenty-one eviscerated rats were given a glucose load of 44/100/h with 4 units of insulin per 24 hours per rat. The average survival was 2194 ± 75 minutes. The standard deviation of the difference (366) between the averages was 137 giving a ratio of 2.7 between the difference and its standard deviation, thereby indicating a statistical probability of 99 chances in 100 that the average survival of the insulin treated rats was greater than the average survival of the rats not treated with insulin.

When the survival times for the insulin

series of both experiments were averaged together and the survival times for the no-insulin rats were averaged together, the standard deviation of the difference (316) in averages was 54 giving a ratio of 5.85 between the difference and its standard deviation, thereby indicating that the direction of the difference in averages was highly significant from the standpoint of statistical probability.

Discussion. This is one of a series of studies on factors which influence the survival of eviscerated rats. The times of survival can be prolonged by the continuous intravenous injection of saline only;² the addition of glucose without insulin prolongs survival and the administration of higher loads of glucose with insulin gives further prolongation of average survival times. The longest survival of any animal in these experiments was 44 hours and 4 minutes.

Summary. Eviscerated rats were given continuous intravenous infusions of glucose in saline. The addition of insulin and glucose prolonged the survivals of these animals to a greater extent than did glucose without insulin.

² Ingle, D. J., Sheppard, R., and Winter, H. A., *Am. J. Physiol.*, 1945, 144, 255.

16748

Effect of Diethyldithiocarbamate on the Respiration of Active and Blocked Embryonic Cells.*

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The action of urethanes (carbamates) on respiration and related phenomena of living cells, in general, shows a marked stimulation with low doses and a corresponding definite inhibition with higher concentrations.¹ The

* Aided by grant from the National Institute of Health. Acknowledgment is gratefully made to Etta Andrews, John Johnston, and Herman Tharp for technical assistance in carrying out these experiments.

¹ Fisher, K. C., and Henry, R. J., *J. Gen. Physiol.*, 1943, 27, 469.

efficiency of homologous series of these compounds in producing these reactions increases markedly with their increase in carbon content.² Recent results concerning their carcinogenic activity add further interest to an elucidation of their basic reactions upon living cells and tissues.³ The present report is concerned with results of experiments upon

² Taylor, G. W., *J. Cell. and Comp. Physiol.*, 1936, 7, 409.

³ Larsen, C. D., *J. Nat. Cancer Inst.*, 1947, 8, 99.

3. In general, diethyldithiocarbamate produces stimulation in low doses and in high doses an initial inhibition followed by a marked stimulation in the oxygen intake of embryonic cells.

4. Partial recovery from high initial doses of the reagent occurs.

5. Copper salts are markedly antagonized when in the presence of the carbamate.

16749

Alkaline Phosphatase of the Serum in Experimental Lathyrism of the White Rat.

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Lathyrism, a condition associated with the consumption of considerable amounts of legumes of the genus *Lathyrus*, notably *Lathyrus sativus*, *claymenum*, and *cicera*, is a disease of frequent occurrence in man in certain regions of India, North Africa and Spain.¹⁻³ It has also been observed in domestic animals although marked species variation in susceptibility to the intoxicant has been noted. The causal agent, a toxic component of the seed, has not been isolated, nor is the mechanism of the production of the disease known. The intoxicant appears to act as a neurotoxin.⁴

Recently Rudra and Bhattacharya⁵ have reported a marked increase in serum alkaline phosphatase content in human lathyrism. Ten male patients, ranging in age from 16 to 45 years were studied. Neither the duration nor the intensity of the disease was stated, although it was observed that "usually the serum-phosphatase level is a measure of the

severity of the disease." In the group, the enzyme content of the serum ranged from 18.7 to 56.5 units (Bodansky), with an average of 38.7. In a group of 12 healthy males of the same age range, the phosphatase content varied from 0.8 to 5.7 units. The minimal value of the lathyrism group was 228% higher than the maximal value of the normal group.

Experimental lathyrism has been produced in young rats fed diets which contained considerable amounts of the seed of the flowering sweet pea (*Lathyrus odoratus*)^{6,7} and other species of *Lathyrus*. In view of the marked increases in alkaline phosphatase of the serum reported in human lathyrism, we have studied the enzyme content of the serum in experimental lathyrism of the white rat.

In preliminary experiments with young (50 g) rats, difficulty was experienced in obtaining amounts of serum sufficient for serial determinations during the course of the experiments. Subsequently we have employed somewhat older animals in which it was possible to secure repeated samples of blood for analysis as the symptoms of lathyrism developed. With older animals, however, the onset of lathyrism is greatly delayed and the

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¹ Schuchardt, B., *Deut. Arch. klin. Med.*, 1886-87, 40, 312.

² Stockman, R., *J. Pharm. Exp. Therap.*, 1929, 37, 43.

³ Diaz, C. J., *Revista clin. Español*, 1941, 3, 303.

⁴ Filimonoff, I. N., *Z. ges. Neurol. Psychiatr.*, 1926, 105, 76.

⁵ Rudra, M. N., and Bhattacharya, K. P., *Lancet*, 1946, 1, 688.

⁶ Geiger, B. J., Steenbock, H., and Parsons, H. T., *J. Nutrition*, 1933, 6, 427.

⁷ Lewis, H. B., Fajans, R. S., Esterer, M. B., Chen, C.-W., and Oliphant, M. J., *Nutrition*, 1948, 36, 537.

interest to consider such a reaction as a possible way of throwing light upon the manner of its biological action. Copper acetate alone, when added to embryos produces a rather marked toxic effect (Fig. 3). Embryos exposed to the carbamate and then subjected to a similar concentration of copper acetate, show a rather marked decrease in the effects of the added copper. Such a result is probably due to the copper carbamate compounds formed which in turn are much less soluble and less toxic than the pure copper acetate. Whether the copper is added during the inhibitory or stimulating phase of the carbamate effect seems to make little difference in the results produced. This rather rapid and definite binding of copper by this reagent suggests that in its action upon the respiratory mechanism of embryonic cells some similar reaction may possibly occur. That sulfhydryl-containing com-

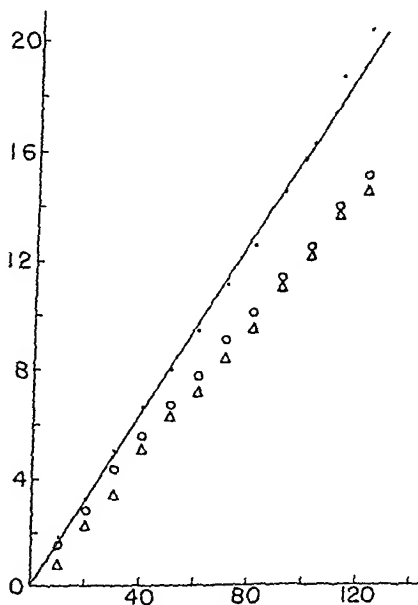


FIG. 2.

Shows recovery curves for embryos treated with diethyldithiocarbamate for 30- and 60-minute periods. Abcissa, time in minutes; ordinate, total oxygen (mm^3) per 100 embryos. Solid circles, control embryos in Ringer solution. Open circles, similar embryos treated for 60 minutes in 0.0166 M diethyldithiocarbamate, removed, washed in Ringer and oxygen uptake determined. Triangles, embryos treated for 30 minutes with reagent and treated as for open circles.

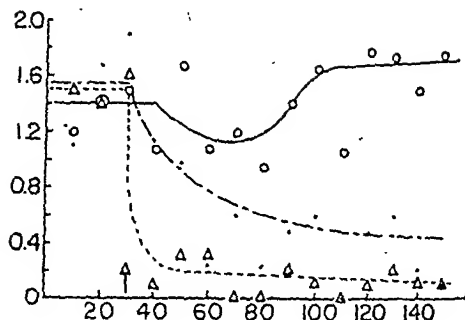


FIG. 3.

Shows the effect of additions of copper acetate and diethyldithiocarbamate upon the oxygen consumption of active cells of embryo. Abcissa, time in minutes; ordinate, $\text{mm}^3 \text{O}_2$ per 100 embryos per 10 minutes. Arrow indicates time of addition of reagent from the sidearm into flask. Open circles, embryos in 0.0166 M diethyldithiocarbamate, Ringer solution added from sidearm. Triangles, embryos in Ringer solution, copper acetate added from sidearm (final conc. = 0.008 M). Solid circles, embryos in 0.0166 M diethyldithiocarbamate, 0.0252 M copper acetate added from sidearm (final conc. = 0.008 M).

pounds have especial affinity for copper-bearing enzymes seems well established.⁶

A significant feature in the effects of diethyldithiocarbamate on the oxygen intake of embryonic cells is the striking difference in its responses compared with those for other carbamates such as ethyl carbamate (urethane). In the case of the latter, a stimulation occurs in low doses and a marked and constant inhibition in higher doses while for diethyldithiocarbamate low concentrations stimulate but with high concentrations an initial marked inhibition is invariably followed by stimulation. Further investigation seems necessary in order to definitely determine the reason for these basic differences in the reactions of such compounds.

Summary and Conclusion. 1. A study has been made on the effects of sodium diethyldithiocarbamate on the oxygen consumption of mitotically active and blocked embryonic cells of the embryo of the grasshopper, *Melanoplus differentialis*.

2. No significant differences in response of the active and blocked cells to the reagent are noted.

⁶ Barron, E. S. G., and Singer, T. P., *J. Bio. Chem.*, 1945, 157, 221.

Hepatic Inactivation of Testicular Androgen in the White Leghorn Cockerel.*†

E. CRANSTON BERNSTORF. (Introduced by R. T. Hill.)

From the Department of Zoology, Indiana University, Bloomington, Ind.

Both the ovary and testis show a marked hypertrophy when grafted into the spleen of a castrate rat of the same sex.¹⁻³ This hypertrophy apparently occurs because the liver removes from the blood stream the sex hormone produced by the graft and in so doing frees the pituitary from the usual inhibition which the gonads exert upon its secretion of gonadotropin. Heller and Jungck⁴ found hypertrophy of the spleen-grafted ovary to be prevented by subcutaneous administration of estradiol-benzoate.

The fact that the chick is used in a successful method of androgen assay⁵ suggests this animal's use for hepatic inactivation studies.

Experimental. All chicks used in these experiments were single-comb white leghorn cockerels which were allowed to eat standard feed *ad libitum*. Castration, carried out under ether anesthesia, was performed by means of an incision made in the posterior left intercostal space. When a testis graft was made into the spleen, castration was done as described and jeweler's forceps were used to make the autotransplantation. Chicks for these transplants were selected at random and

operated on at the age of 5 and 7 days (series I) and others at the age of 20 or 21 days (series II). At the time of the first operation other chicks were designated as controls.

Each chick was weighed and the comb height and length measured on every fifth day. A comb factor reading was obtained by multiplying the height and length in millimeters and dividing by two $\left(\frac{H \times L}{2}\right)$. This

factor has been found to give a reliable indication of the comb growth changes and thereby an estimation of the amount of sex hormone to which the comb has been exposed. To adjust for differences in body weight the total number of comb factor units (c.f.u.) of each was divided by the average body weight of that group, thus obtaining the average number of c.f.u. per gram body weight for each series.

Results and discussion. Histological study of the spleens showed that the grafts, although not greatly enlarged, had "taken." As mentioned earlier the liver destroys the estrogen or androgen produced by a gonad placed in the spleen of the rat.¹⁻⁴ Data presented in Table I indicates that hormone produced by the spleen-grafted testis of the chick is also partially inactivated by the liver. The comb at all times indicates the amount of hormone which is allowed to pass into the general circulation and shows whether or not the inactivation is complete. Our work suggests use of the chick comb to have a marked advantage over use of the rat for this type of androgen assay; not only can as many measurements be taken as desired, but a sensitive quantitative response is given even to very small amounts of hormone.

The relative amount and effectiveness of the androgen secreted by the controls, as shown

* From a thesis presented to the faculty of the Graduate School of Indiana University in partial fulfillment of the requirements for the degree of Master of Arts in Zoology, June, 1948.

† The help and guidance given by Dr. W. R. Breneman during the course of this study is gratefully acknowledged.

¹ Biskind, M. S., and Biskind, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 176.

² Biskind, M. S., and Biskind, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 4.

³ Biskind, M. S., and Schelsnyak, M. C., *Endocrinology*, 1942, **30**, 819.

⁴ Heller, C. G., and Jungck, E. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 152.

⁵ Breneman, W. R., *Endocrinology*, 1941, **28**, 222.

TABLE I.
Alkaline Phosphatase of Serum of Rats Fed Sweet Pea and Edible White Pea Diets.

Period on diet, days*	Diet Rat No.	Phosphatase units (King-Armstrong)									
		Sweet pea					White pea				
		1	3	5	9	11	2	4	6	10	12
0		85	89	83	87	59	142	82	101	70	62
8-17		76	64	112	70	76	92	90	103	68	75
29-33		80	67	115	70	66	80	129	136	81	109
63		94	73	54	47	99	118	145	67	46	92

* The period at which samples of blood were collected varied somewhat within the ranges indicated for different rats. Rats 1 and 2, 3 and 4, etc., were paired in the feeding experiments.

changes in the bones^{6,7} are less marked.

Ten adult male rats (200 g) were fed the diet described previously⁷ (50% of ground sweet pea seed) for periods of 10 weeks. An equal number of controls, in paired feeding experiments, received the edible white pea of commerce (*Pisum sativum*, var. *arvense*). All animals which received the sweet pea diet developed lameness, spinal curvature, and, in a few cases, marked paralysis during the experimental period. Blood was obtained by clipping the end of the tail after the animal had been placed in a warm chamber at 56° for 5 min. After clotting, the serum was obtained by centrifugation; the centrifugate was decanted and the serum was centrifuged a second time. The upper portion of the second centrifugate was removed by a pipette and used for the phosphatase determination. Since the phosphatase content of white and red cells is much greater than that of the serum, it was necessary to remove all the cellular elements and to avoid hemolysis as much as possible. Alkaline phosphatase was determined by the method of King and Armstrong.⁸ The results are reported as phosphatase units per 100 ml of serum (i.e. mg of phenol liberated by the hydrolysis of disodium

monophenyl phosphate under standard conditions).

Results obtained with 5 pairs of animals are shown in Table I. They are typical of the values obtained with all 10 pairs. Although there was considerable variation in the phosphatase content both in the same rat over the experimental period and also in different animals, there was no evidence of any increase as the clinical symptoms of lathyrism developed, nor was there any significant difference between the values obtained with the control (white pea) and the experimental (sweet pea) groups. Certainly there was no indication of such a marked elevation of the alkaline phosphatase as has been reported by Rudra and Bhattacharya⁵ in human lathyrism.

Summary. No significant changes in the concentration of alkaline phosphatase of the serum were observed in rats in which experimental lathyrism was produced by feeding diets containing 50% of sweet pea (*Lathyrus odoratus*) seed. This does not confirm the observations of Rudra and Bhattacharya in which the phosphatase values were markedly increased in human lathyrism.

We wish to express our indebtedness to the Ferry Morse Seed Company of Detroit for the sweet pea seed and to the Washburn-Wilson Seed Company of Moscow, Idaho, for the edible split pea seed.

⁸ King, E. L., and Armstrong, A. R., *Can. Med. Assn. J.*, 1934, 31, 376.

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operated on at the age of 5 and 7 days (series I) and others at the age of 20 or 21 days (series II). At the time of the first operation other chicks were designated as controls.

Each chick was weighed and the comb height and length measured on every fifth day. A comb factor reading was obtained by multiplying the height and length in millimeters and dividing by two ($\frac{H \times L}{2}$). This

factor has been found to give a reliable indication of the comb growth changes and thereby an estimation of the amount of sex hormone to which the comb has been exposed. To adjust for differences in body weight the total number of comb factor units (c.f.u.) of each was divided by the average body weight of that group, thus obtaining the average number of c.f.u. per gram body weight for each series.

Results and discussion. Histological study of the spleens showed that the grafts, although not greatly enlarged, had "taken." As mentioned earlier the liver destroys the estrogen or androgen produced by a gonad placed in the spleen of the rat.¹⁻⁴ Data presented in Table I indicates that hormone produced by the spleen-grafted testis of the chick is also partially inactivated by the liver. The comb at all times indicates the amount of hormone which is allowed to pass into the general circulation and shows whether or not the inactivation is complete. Our work suggests use of the chick comb to have a marked advantage over use of the rat for this type of androgen assay; not only can as many measurements be taken as desired, but a sensitive quantitative response is given even to very small amounts of hormone.

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² Biskind, M. S., and Biskind, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1945, **50**, 4.

³ Biskind, M. S., and Schelsnyak, M. C., *Endocrinology*, 1942, **30**, 819.

⁴ Heller, C. G., and Jungck, E. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 152.

⁵ Breneman, W. R., *Endocrinology*, 1941, **28**, 222.

TABLE I.
Effect of Intrasplenic Testis Transplants Upon Comb Growth.

	Age 36 days				Age 46 days		
	Control	SI	SII	S 89	Control	SI	SII
Avg body wt	369.0	354.0	345.2	275.9	513.2	503.4	486.0
Avg comb factor units	371.6	251.9	232.2	111.4	839.4	506.3	435.1
Avg comb factor units per g wt	0.995	0.697*	0.671*	0.396*	1.587	0.983*	0.908*
No. in group	16	15	14	12	16	15	14

* T value is significant at 1%. (Snedecor, George W., *Statistical Methods*, The Iowa State Press, Ames, Iowa, 4th ed., 1946.)

SI: chicks caponized at 5 or 7 days of age.

SII: chicks caponized at 20 or 21 days of age.

S 89: chicks caponized at 5 days of age.

by the number of comb factor units, and of that which passed through the liver of the spleen-graft chicks may be seen from Table I. Complete inactivation did not occur, as shown by comparison of series I and II with the chicks of series 89 (caponized controls), which were operated on at 5 days of age and otherwise given similar treatment. It is also seen that the number of c.f.u./g body weight in series I is larger than that in series II. This is explained by the fact that the transplants in series I had a longer time to grow and secrete than did those of series II grafted 2 weeks later. This explanation is supported by the fact that the difference in number of c.f.u./g body weight for series I and II at 46 days (7.6%) is greater than that at 36 days (3.7%). The comparison at 36 days shows that enough hormone had passed the liver to augment comb growth by at least 40.9%.

The fact that in other animals incomplete

inactivation of endogenous hormone has not been reported under these conditions suggests either that inactivation is different in the chick or that the chick permits the detection of this minute quantity of hormone. The comb shows a progressive cumulative change which makes possible the measurement of a slight failure of inactivation occurring over an extended period of time. Although at sacrifice some testicular material was found in the normal testis location in a few birds, the data indicate that its effect was insignificant.

Summary. These data show that: (1) the testis hormone is partially removed from the circulation by the liver of the chick, and that (2) the comb gives a sensitive indication of the extent of hepatic inactivation of androgens. This may justify speculation that some androgenic activity may be retained by the products of hepatic metabolism.

16751

Excretion of Adrenal Corticoids in the Sweat.

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From the Laboratory of Applied Physiology, University of North Carolina, Chapel Hill.

In experiments on the urinary excretion of adrenal corticoids in strenuous exercise it was noted that, with the onset of hot weather, the excess steroid excretion in the urine was strikingly reduced. It occurred to us that this might be due to the excretion of steroids in

the sweat and the following study was carried out.

Two healthy adult subjects ran on a motor-driven treadmill at speeds of 8 to 12 miles per hour and a grade of 10% for periods of 30 to 45 minutes. The running was interrup-

ted every 2 or 3 minutes for a brief rest period. The room temperature ranged from 80 to 90°F., and the relative humidity from 60 to 90%. The total amount of sweat produced during an experiment varied from 550 to 700 g as estimated from the loss in body weight. Sweat was collected by allowing it to drip from the elbows into large funnels which drained into graduated cylinders. Contamination of the sweat by cellular debris was minimized by vigorous scrubbing of the arms before the experiment. When 50 ml of sweat was collected it was filtered and analyzed for adrenal corticoids by the method of Heard *et al.*^{1,2} According to these authors, the method measures reducing steroids of the adrenal cortical hormone class, containing a primary or secondary (but not tertiary) α -ketol function, an $\alpha\beta$ -unsaturated 3-ketone group, or both. The adrenal cortex is considered by them to be the major source of these steroids.

TABLE I.
Excretion of Adrenal Corticoids in the Sweat.

Subject	Adrenal corticoid concentration (γ per 100 ml)	
	Exercise sweat	Thermal sweat
J.N.	80	72
"	76	64
"	76	40
"	80	52
"	44	60
"	46	
"	40	
"	56	
"	40	
"	40	
W.P.	48	
"	60	
"	64	
"	46	
"	50	
Mean	56.4	57.6

In another series of experiments, the concentration of adrenal corticoids in thermally-induced sweat was determined; this sweating was induced by partial immersion of the body in a hydrotherapy whirlpool bath at $112 \pm 3^\circ\text{F}$ for 30 minutes.

Results. The results are shown in Table I. It is apparent that relatively large amounts of adrenal corticoids are excreted in the sweat induced by exercise and thermal stimulation.

Discussion. Increased urinary excretion of adrenal corticoids occurs during response of the body to many types of stress³⁻⁵ and is believed to indicate increased activity of the adrenal cortex. Hoagland,³ using the method of Heard and Sobel for adrenal corticoids and a method specific for 17-ketosteroids, found a rise in the urinary output of both groups of substances after intramuscular injection of adrenal cortical extract. Since the rate of excretion of adrenal corticoids in sweat during exercise and thermal stimulation in our experiments exceeded the resting rate of renal excretion it is a reasonable assumption that it indicates an activation of the adrenal cortex in response to the stresses of heat and strenuous exertion. Our results also emphasize the fact that in stresses associated with sweating, a significant fraction of the total corticoid excretion is accounted for in the sweat.

Conclusion. There is a significant excretion of adrenal corticoids in sweat induced by exercise and thermal stimulation.

We are indebted to Dr. Frederick F. Yonkman of the Ciba Pharmaceutical Products, Inc., for the supply of desoxycorticosterone used to establish the adrenal corticoid calibration curve and to Mr. Walter E. Pupa for serving as a subject in some of the experiments.

¹ Heard, R. D. H., and Sobel, H., *J. Biol. Chem.*, 1946, 165, 687.

² Heard, R. H. D., Sobel, H., and Venning, E. H., *J. Biol. Chem.*, 1947, 165, 699.

³ Hoagland, H., *J. Aviation Med.*, 1947, 18, 450.

⁴ Selye, H., *J. Clin. Endocrinology*, 1946, 6, 117.

⁵ Venning, E. H., and Kazmin, V., *Endocrinology*, 1946, 30, 131.

Influence of Histamine on Adenosine Triphosphatase.

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In all snake venoms hitherto investigated a powerful ATP-ase (ophio-ATP-ase) has been found, which is activated by magnesium, calcium and cobalt ions and inhibited by zinc ions.^{1,2} Histamine and pyridoxamine act as inhibitors, epinephrine as activator.

The question arose as to whether similar reactions take place in the animal tissues and thus contribute to the understanding of the intimate action of histamine on many organs. Therefore homogenates of different organs were tried on their ability to split ATP. Similar conditions as with ophio-ATP-ase were used (Table I).

Three ATP-ases are known to occur in animal tissues. The ATP-ases from electric tissues of fish³ and from mammal muscle⁴ are activated by magnesium and inhibited by calcium, while the ATP-ase that is inseparable from myosine is influenced by these ions in the opposite way.⁵

In the lung, gastric mucosa, adrenal and kidney of guinea pigs, and in the cortex and medulla of adrenals of cattle an ATP-ase is present which is activated by magnesium, calcium and cobalt ions. The activation by

magnesium is the highest and sometimes reaches values which are 10 times higher than the original ones. 0.00001-molar magnesium chloride still caused a rise in ATP-ase activity. By these reactions this ATP-ase differs from the above-mentioned 3 ATP-ases of animal tissues, but resembles the ophio-ATP-ase. The identity is not complete, since zinc ions accelerate the reaction velocity of the new ATP-ase.

The reaction does not stop completely after the liberation of one molecule of phosphoric acid, but the reaction velocity drops after reaching this point. These and other results lead to the assumption that the second molecule of phosphoric acid is split off by other enzymes. This question needs further elucidation.

The new ATP-ase is affected by histamine and epinephrine in a similar way as is ophio-ATP-ase. The highest degree of inhibition is usually reached in the presence of a magnesium concentration that is below the optimum. Under certain conditions calcium exactly counterbalances the influence of histamine (Table I).

TABLE I.
ATP-ase of the Adrenals of the Guinea Pig.
ATP, 0.0015-molar; glycine buffer, pH 8.3; homogenate corresponding to 1 mg of tissue;
volume, 1 ml; incubation period, 15 min.

	Without inorganic ions, Qp*	With 0.001-molar magnesium, Qp	With 0.001-molar calcium, Qp
Without amines	10.4	60.0	25.2
.001-molar histamine	3.6	39.6	11.2
.001-molar epinephrine	20.0	73.6	34.8

* Qp = μ g of inorganic phosphorus per mg of fresh tissue per hr.

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¹ Zeller, E. A., *Experientia*, 1948, 4, 194.

² Zeller, E. A., Physiology of Snake Venom Adenosine Triphosphatase. Unpublished data.

³ Greville, G. D., and Lehmann, *Nature*, 1943, 152, 81.

⁴ Kielley, W. W., and Meyerhof, Otto, *J. Biol. Chem.*, 1948, 174, 387.

⁵ Engelhardt, V. A., Adenosinetriphosphatase Properties of Myosin. In: *Advances in Enzymology*. New York, Interscience Publishers, Inc., 1946, vol. 6, pp. 147-191.

Pyridoxamine acts like histamine, while aliphatic diamines activate rather than inactivate the tissue-ATP-ase and ophio-ATP-ase.

By these results histamine and ATP, which

have been connected with the mechanism of shock production, are brought in a close relationship.

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Failure to Produce Lesions or Auto-Antibodies in Rabbits by Injecting Tissue Extracts, Streptococci and Adjuvants.*

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Schwentker and Comploier¹ demonstrated the formation of complement fixing antibodies for homologous kidney tissue in rabbits which had been inoculated with a mixture of kidney tissue extract and staphylococcal or streptococcal toxin. Cavelti and Cavelti² subsequently reported that an antibody for kidney tissue could be demonstrated by the collodion particle-agglutination technic in rabbits and rats which were inoculated with a mixture of homologous tissue and Group A streptococci. The latter workers also reported that rats immunized in this fashion developed acute and chronic glomerulonephritis. Recently, Cavelti³ described the development of antibody for homologous heart tissue in rats injected with heart extracts and streptococci, and also demonstrated the appearance of inflammatory myocardial lesions in these animals.

Kabat, Wolf and Bezer⁴ and Morgan⁵ have recently confirmed the earlier observation of

Rivers and Schwentker⁶ that the immunization of monkeys with extracts of homologous brain tissue leads to the formation of demyelinating lesions of the central nervous system which resemble those seen in multiple sclerosis. These lesions were brought about in greatly accelerated fashion by the use of the adjuvants described by Freund,⁷ consisting of "Falba" or "Aquaphor", paraffin oil, and killed tubercle bacilli.

In the present study, the effect of inoculating rabbits with homologous heart and kidney tissue in combination with streptococci and Freund's adjuvants was studied.

Material and methods. *Rabbits.* Hybrid brown and grey male rabbits, weighing between 1.8 to 2.3 kg each, were used in all experiments.

Tissue Suspensions. Heart and kidney tissue were obtained from normal rabbits. The organs were thoroughly perfused with physiological saline under sterile conditions before removal, and were either used immediately or stored whole in a CO₂ ice box. Antigens were prepared by grinding portions of each organ with abrasive, suspending in sufficient physiological saline to make a 10% suspension, and partially clearing by centrifugation for 5 minutes at 1500 R.P.M. Bacteriological cultures were made from samples of each suspension to ascertain sterility.

Streptococci. Several strains of beta hemo-

* This work was supported by a grant from the Life Insurance Medical Research Fund.

[†] Senior Pediatric Fellow of the National Research Council.

¹ Schwentker, F. F., and Comploier, F. C., *J. Exp. Med.*, 1939, **70**, 223.

² Cavelti, P. A., and Cavelti, E. S., *Arch. Path.*, 1945, **40**, 158.

³ Cavelti, P. A., *Arch. Path.*, 1947, **44**, 1.

⁴ Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, **85**, 117.

⁵ Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131.

⁶ Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.*, 1935, **61**, 689.

⁷ Freund, J., and McDermott, K., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 548.

lytic streptococci were employed: a group C strain (H46A) obtained through the courtesy of Dr. O. D. Ratnoff, and 3 Group A strains (type 19) recovered from the throat cultures of patients with acute tonsillitis (one of whom also had rheumatic fever). Eighteen-hour cultures of these organisms were grown in trypticase-soy broth, after which the streptococci were killed by heating at 56°C for 45 minutes.

Adjuvants. Heat-killed, dried tubercle bacilli, obtained through the courtesy of Dr. Jules Freund, were suspended in paraffin oil to a concentration of 0.4 mg per cc. Three parts of this suspension were transferred slowly, with constant stirring, to 2 parts of melted "Falba". The resultant mixture was immediately combined with other reagents as indicated below.

Preparation of Antigen Mixtures. Equal volumes of tissue suspension and streptococcus culture were mixed together, and 2 volumes of this mixture were added, drop by drop, to 5 parts of the adjuvant, with constant stirring. In control experiments, sterile broth replaced the streptococcus culture. Microscopic examination of each final suspension was made in order to ascertain that uniform globules of small size had been obtained.

Inoculation of Animals. Each rabbit was given 4 injections of 1 cc each, in separate subcutaneous areas. The injections were repeated at weekly intervals for a total of 3 weeks.

Each rabbit was bled from the ear before the first injection, and at 2-week intervals following the final injection, and the serum was stored in the frozen state. Sample animals were sacrificed in each experiment at 2, 4, 6, 8, and 10 weeks following the final injection of antigen, and portions of heart, kidney and other organs were placed in 10% formalin for subsequent histological examination.

Complement Fixation Tests. Tissue antigens for the complement fixation test consisted of 5 or 10% suspensions of heart or kidney tissue in physiological saline, which were made up on the day of testing and clarified by cen-

trifugation at 2000 R.P.M. for 10 minutes. In some experiments, modified antigens were prepared by subjecting the tissue suspensions to a) centrifugation at 12,000 R.P.M. for 30 minutes, b) heating at various temperatures between 45°C and 80°C, and c) exposure to various pH levels between 4.5 and 9.5. The standard complement fixation test was employed, using 0.2 cc of complement (two units), 0.2 cc of the antigen, 0.2 cc of the serum under test, and, after a period of two hours at 37°C, 0.6 cc of sensitized sheep cells consisting of 0.2 cc of rabbit amboceptor (two units) and 0.4 cc of 2% cells. Fixation of complement was interpreted as occurring in those tubes which showed no hemolysis after 30 minutes in a 37°C water-bath.

Collodion Particle Agglutination Test. Collodion particles were suspended in normal rabbit heart and kidney extracts by the method described by Cavelti.⁸ The agglutination of particles in varying dilutions of serum was tested by the method of the same author.

Results. Approximately 350 rabbits have been subjected to immunization with homologous tissue suspensions in combination with streptococci and Freund's adjuvants. Group C beta hemolytic streptococci were employed in the antigens used for one third of this group, and Group A streptococci in the remainder. In addition, a smaller number of control animals were injected with tissue suspensions alone, or with the suspensions plus adjuvants.

Antibody Determinations. As has been shown by Kidd and Friedewald,⁹ normal rabbit serum contains a substance which causes fixation of complement with tissue suspensions from various rabbit organs. This "antibody", which is readily inactivated by heating at 65°C for 30 minutes, was encountered in most of the sera employed in this study, in titers of 1:16 or 1:32. In no instance, however, was a significant rise in complement fixation titer encountered between the pre-inoculation sera and the sera obtained at any period following

⁸ Cavelti, P. A., *J. Immunol.*, 1944, **49**, 365.

⁹ Kidd, J. G., and Friedewald, W. F., *J. Exp. Med.*, 1942, **76**, 543.

inoculation, with any of the antigens employed. Heating the sera at 65°C resulted in the disappearance of complement-fixing antibody from all sera tested. Treatment of the tissue antigens by high-speed centrifugation, heating, or exposure to different pH levels did not result in any positive complement fixation reactions.

The collodion particle technic yielded results which were uniformly negative in all experiments. No evidence of an antibody for heart or kidney tissue was demonstrable in the sera of rabbits following immunization with any of the materials employed.

Pathological Studies. No significant cardiac or renal lesions were demonstrable in any of the rabbits which were injected with tissue suspensions, adjuvants and Group C streptococci. The results obtained with Group A streptococci were also negative, with the exception of a single experiment involving twenty-one rabbits. In this experiment, 3 animals received kidney tissue suspension in combination with Group A streptococci and Freund's adjuvants, 3 received the tissue suspension with streptococci but without adjuvants, 3 received the tissue suspension with the adjuvants but without streptococci, and 3 received the adjuvants and streptococci without tissue suspension; a similar group of rabbits were given a suspension of heart tissue in the same combinations. The animals were sacrificed at the end of the sixth week after inoculation. All of the rabbits which received either heart or kidney tissue in combination with streptococci and adjuvants showed extensive areas of acute inflammatory reaction and muscle fiber destruction involving the right ventricle. These lesions did not resemble the characteristic lesion of rheumatic myocarditis. Similar but less extensive lesions were seen in 2 of the rabbits which were given the mixture of kidney suspension and streptococci without adjuvants, and in 2 of the rabbits which received heart suspension and streptococci without adjuvants. No lesions occurred in the hearts of the rabbits receiving tissue suspensions with adjuvants alone, or in the group receiving streptococci and adjuvants without tissue suspensions. No

significant kidney lesions were seen in any of the experimental animals.

Many attempts were made to repeat these observations, employing the same strain of streptococcus and, so far as possible, the same materials and experimental conditions. All of these attempts were unsuccessful. No myocardial lesions which were in any way similar to those observed in the 10 rabbits referred to above were encountered again. Occasional small areas of infiltration by round cells were encountered between muscle fibers, but these seen with equal frequency in the heart tissue of normal untreated rabbits.

No explanation can be given for the myocardial inflammatory lesions in the single experiment described above. Although bacteriological cultures of the heart tissue and blood of these animals were negative, and the injection of serum from these rabbits into normal animals failed to produce any evidence of disease, it is considered possible that the myocardial lesions may have been due to an irrelevant infection of these animals by an unknown virus or bacterium. In view of the repeatedly negative results which were obtained when the experiment was duplicated, the significance of the myocardial lesions is doubtful.

Summary. The inoculation of rabbits with suspensions of homologous kidney or heart tissue, in combination with heat-killed cultures of Group A and Group C beta hemolytic streptococci and Freund's adjuvants, did not bring about the formation of detectable antibodies for homologous tissue.

Rabbits inoculated in this manner did not develop myocardial or renal lesions suggestive of rheumatic myocarditis or glomerulonephritis. In experiments involving approximately 350 rabbits, the only significant pathological finding consisted of acute inflammatory lesions in the myocardium of the right ventricle which occurred in ten animals. The latter animals had received injections of homologous heart and kidney tissue in combination with Group A streptococci and the adjuvants. The lesions did not resemble rheumatic myocarditis. Repeated attempts to confirm this observation, under similar conditions, yielded uniformly negative results.

Effect of Oxythiamine on Infection of Mice with the Lansing Strain of Poliomyelitis Virus.*

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It was reported from this laboratory¹ that a dietary deficiency of thiamine increased the resistance of albino mice to the Lansing strain of poliomyelitis virus. A few months later Rasmussen, *et al.*² reported similar observations. Using Theiler's virus. Lichstein and associates in the same laboratory³ found that deficiencies of some other dietary constituents afforded a similar degree of protection to mice.

However, none of the several other dietary deficiencies studied at The Children's Hospital of Philadelphia showed a degree of protection against the Lansing strain, which is considered a true poliomyelitis virus, equaling that of a deficiency of thiamine, although these included some of those reported by Lichstein as giving protection against Theiler's virus.

Because of the superior effect of a deficiency of thiamine in mice inoculated with the Lansing strain, a study of the influence of inhibitory analogues of thiamine on the resistance of mice to this virus was indicated.

The first thiamine analogue thoroughly

studied in this laboratory was oxythiamine.[†] It is produced by replacing the NH₂ group on the pyrimidine ring of thiamine by an OH group. Some of the physiological properties of this analogue have been studied by Soodak and Cerecedo.⁴ They found that mice receiving 1 mg of thiamine per day and given 25, 50 or 100 mg of oxythiamine daily, lost weight rapidly and died in about 2 weeks. They also showed that the enzyme of carp which destroys thiamine was inhibited by oxythiamine, but they did not determine the minimum amount of thiamine which would just off-set a given quantity of oxythiamine.

Several experiments were performed in the authors' laboratory studying the effect of this inhibitor of thiamine upon the resistance of mice to the virus of poliomyelitis. All of these gave the same general results. It is sufficient, therefore, to present in this paper the data obtained in the largest and most complete of the experiments.

Experimental. Virus. The virus and the technics employed were described in an earlier paper.⁵ A recent test in this laboratory showed that the virus was still infectious for rhesus monkeys.

Animals. The Swiss white mice used in these studies came from the same colony that has been maintained in this laboratory for 14 years, and has produced mice for all of the resistance experiments conducted here.

The usual precautions described elsewhere⁶ and essential for this type of experiment were

* Aided by a grant from the National Foundation for Infantile Paralysis.

¹ Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 215.

² Rasmussen, A. F., Jr., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., *J. Bact.*, 1943, **45**, 85.

³ Lichstein, H. C., McCall, K. B., Kearney, E. B., Elvehjem, C. A., and Clark, P. F., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 279.

[†] We gratefully acknowledge the advice and co-operation of Dr. Gustav J. Martin of the National Drug Company of Philadelphia, Pa., who supplied the oxythiamine and provided the data on its structure, purity and microbiological assay.

⁴ Soodak, M., and Cerecedo, L. R., *J. Am. Chem. Soc.*, 1944, **66**, 1988.

⁵ Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, **79**, 221.

⁶ Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, **80**, 257.

taken. These included split litter technic for distribution, temperature and humidity control of the experimental rooms, and even a random distribution of the animals in the room to avoid the effect of any environmental differences. The mice were weighed daily, and following inoculation, each animal was removed from its cage for examination every 6 hours, day and night.

Diet. The basal complete diet had the following composition expressed in %: Casein, crude 25.0, cellulose, 2.0, salt mixture,⁷ 4.0, linseed oil, 1.5, wheat germ oil, 1.0, glucose (cerelose) 66.5, carotene concentrate in oil, 5 drops, (Wyeth - 275 U.S.P. units pro-vitamin A per drop) Drisdol, 0.4 drop (Winthrop-250 U.S.P. units vitamin D₂ per drop). The B vitamins were supplied in the following quantities (mg per 100 g of diet.): Thiamine chloride 0.2, riboflavin 0.5, pyridoxine 0.5, calcium pantothenate 5.0, nicotinic acid 10.0, inositol 10.0, *p*-aminobenzoic acid 10.0, choline chloride 100.

This diet was given to the complete-diet control groups and to all of the oxythiamine groups. It was modified in the two low-thiamine groups only in so far as the thiamine content was concerned. Before the experimental regimens were initiated, all the animals were put on the complete diet for 2 days after they had been distributed into their individual experimental jars, in order to accustom them to the purified diet.

Inhibition Index.[†] Preparatory to the experiment, the inhibition index of oxythiamine was evaluated at 3 levels of thiamine: 3, 6, and 12 μ g per mouse per day. In these titrations, both the thiamine and the oxythiamine were administered by mouth from a pipette. The inhibition index for the sample of oxy-

thiamine used in the experiment reported here was found to be about 3 at all 3 levels of thiamine. Using *Lactobacillus fermentum*, the index in Dr. Martin's Laboratory was found to be 10, which is of the same general order as that obtained with the mice.

In the actual experiment this inhibition index was used as a guide, although the conditions were not quite comparable since the thiamine was given as an integral part of the diet, while the oxythiamine, on the other hand, was dissolved in saline and administered by pipette as above. In order to calculate, on the basis of the inhibition index, the amount of oxythiamine necessary to establish and maintain a given state of deficiency, the following assumptions had to be made. If the food intake for a normal mouse is 3 g per day, then each animal would receive 6 μ g of thiamine. As the deficiency state advances under the influence of oxythiamine, the accompanying anorexia leads to a lower intake of diet and correspondingly a lower intake of thiamine. The oxythiamine dosage then has to be decreased by an estimated amount. It is worth commenting that with the pure samples of the analogue, severe signs of deficiency can be quickly terminated by reducing the oxythiamine dosage or by adding a suitable amount of thiamine.

Experimental Groups. The experimental animals were divided into 12 groups as presented in Table I. The mice of 6 of the groups were inoculated with a suspension of mouse brain infected with the Lansing strain of poliomyelitis virus (V), and for each of these there was a control group, the animals of which were injected with a suspension of uninfected ("normal") mouse brain (N).

The first pair of groups (1-V and 1-N) were fed the complete basal diet; the next 4 pairs of groups (2-V and 2-N, 3-V and 3-N, 4-V and 4-N, 5-V and 5-N) received the same complete basal diet and in addition were given oxythiamine; the last pair of groups (6-V and 6-N) were maintained on a low-thiamine diet, in which the intake of thiamine was varied between 20 and 60 μ g per 100 g of diet. The amount of thiamine was regulated in an effort to maintain a level of deficiency that

⁷ Jones, J. H., and Foster, C., *J. Nutr.*, 1942, 24, 245.

[†] The inhibition index is defined as the quotient of the molecular amount of analogue divided by that molecular amount of metabolite which just counteracts the analogue. For example, if the inhibitory action of 1.0 millimole of analogue were just offset by 0.001 millimole of metabolite, then the index would be 1.0/0.001 or 1000 (see D. W. Woolley, *Advances in Enzymology*, 1946, 6, 129.)

TABLE I.

Group No.	No. of mice	Thiamine, mg per 100 g diet	Oxythiamine	Time of beginning oxythiamine in respect to day of inoculation	No. of mice in N-groups dying during experiment
1-V*	56	.2	—	None	—
1-N†	6	.2	—	"	0
2-V	57	.2	+	9 days before	—
2-N	21	.2	+	9 " "	2
3-V	57	.2	+	2 " "	—
3-N	21	.2	+	2 " "	1
4-V	60	.2	+	day of inoculation	—
4-N	21	.2	+	" " "	1
5-V	60	.2	+	4 days after	—
5-N	22	.2	+	4 " "	1
6-V	58	.03-.06	—	None	—
6-N	21	.02-.06	—	"	0

* Groups with "V" in number received virus-infected brain.

† " " " "N" " " " " uninfected brain.

would keep the average weight more or less constant.

The 4 pairs of groups that received the oxythiamine differed among themselves in respect to the day the administration of the analogue was begun. An attempt was made with the first pair of these groups (Group 2-V and 2-N) to produce the deficiency gradually, and to time it so that the animals would be in the same state of deficiency on the day of inoculation as the mice on the diet low in thiamine. To that end 32 μ g of oxythiamine, or about twice the amount that would be counteracted by the thiamine in the diet, were given per animal per day, starting 9 days before inoculation. As that was insufficient to produce a deficiency state in the desired time, the oxythiamine was increased to 64 μ g 3 days later, but after 5 days on this amount it was necessary to reduce it to 8 μ g and then to 4 μ g in order to stop the rapid loss of weight of the animals. After 2 days on 4 μ g and 2 additional days on 8 μ g, their weights could then be held about stationary on 16 μ g per mouse per day.

Groups 3-V, 4-V, and 5-V and their controls were given oxythiamine starting respectively 2 days before inoculation, on the day of inoculation, and 4 days after inoculation. In each of these cases, the dose of oxythiamine was 128 μ g per mouse for the first and second days. This large dose produced an almost immediate fall in weight. From the third day on the dose was gradually reduced, and in

each case finally levelled off at 16 μ g per day.

The experimental animals were observed until the 28th day after inoculation when the experiment was terminated.

Results. Table I shows that there were very few deaths among the groups of mice receiving the "normal" brain. This indicates that the "deficiencies" were not responsible for many deaths in the groups inoculated with virus. This assumption is substantiated by the fact that paralysis or some other sign of poliomyelitis was seen in nearly all animals of the infected groups that died. The following discussion of data is concerned, therefore, only with the animals that were given virus.

The results are presented in Chart I. The curves show the daily cumulative deaths in per cent as ordinates plotted against days after inoculation, as abscissae. It can be seen that, except for a few days at the beginning of the experiment and again at the end, the curve for the animals on the complete diet was above that for any of the "deficient" groups. The curves for Groups 2-V, 3-V and 4-V are very much alike. In these groups, the maximum difference in percentage deaths between Group 1-V and each of the "deficient" groups came at about the 14th day after inoculation. At this point deaths in the "deficient" groups were roughly 50% of those in the group on the complete diet. The death rate in Group 5-V (animals given initial dose of oxythiamine 4 days after inoculation) was nearly equal to

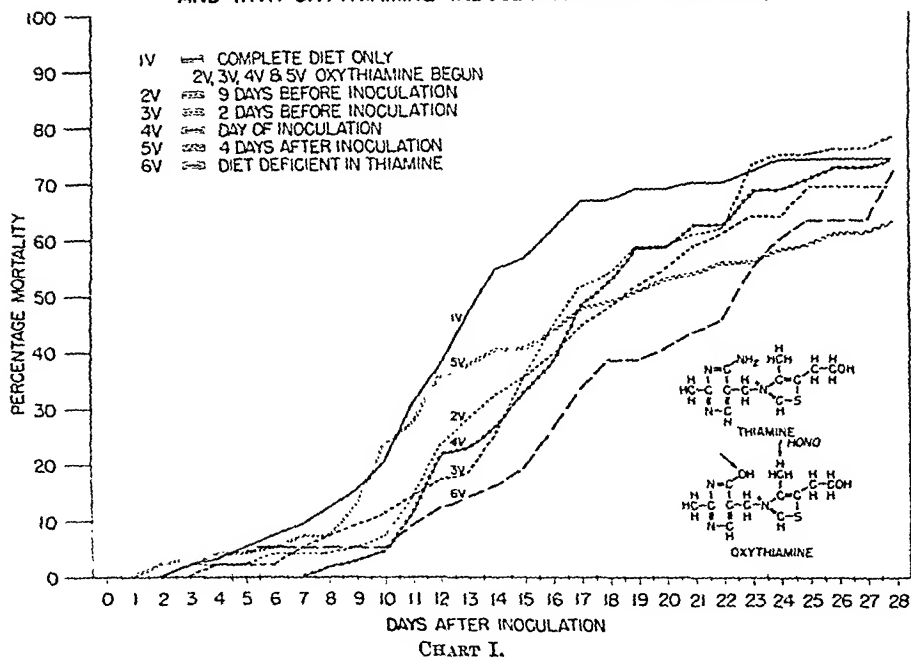
COMPARISON OF COMPLETE DIET WITH LOW THIAMINE DIET
AND WITH OXYTHIAMINE-INDUCED THIAMINE DEFICIENCY

CHART I.

that of Group 1-V for the first 12 days. From then until the end of the experiment the death rate in Group 5-V was considerably decreased, so that even when the experiment was terminated, the percentage deaths was below those of the controls (63 to 74).

None of the groups of mice receiving the oxythiamine were protected to the extent of the animals on the low-thiamine diet. The cumulative percentages of deaths of each virus-inoculated test group were compared by the Chi-square method with the corresponding values for the animals of the virus-inoculated control group for each day of the experiment. The days after inoculation (inclusive) on which the differences were significant are as follows: Group 2-V, 10 to 15; Group 3-V, 12 to 18; Group 4-V, 6 to 17; Group 5-V, 17 to 20; and Group 6-V, 10 to 23.

Discussion. It seems to be possible to induce in mice a state of comparative resistance to poliomyelitis by means of an agent that can be given by mouth, the dose of which can be regulated within certain fairly narrow limits, and the deficiency signs quickly obliterated by suitable doses of thiamine. The protection resulting from oxythiamine treatment appeared to be of the same general order as that resulting from a diet deficient in thiamine, and presumably was associated with the presence of this induced deficiency. As in other deficiency studies, the protective action manifested itself mainly by a slowing of the death rate—a delaying rather than an outright protective action. However, as the animals of group 5-V were given some protection to the end of the experiment, it suggests that if the proper conditions could be found, it is possible that the degree of protection might be increased. It is of particular interest that in this same group (5-V), protection was afforded even though oxythiamine was not administered until 4 days after inoculation with the virus.

The difference between the greater protective effect of thiamine deficiency resulting from a low-thiamine diet, in contrast to the lesser protection afforded by the thiamine deficiency induced by oxythiamine, is not entirely explicable. One of the factors may have

ated by suitable doses of thiamine. The protection resulting from oxythiamine treatment appeared to be of the same general order as that resulting from a diet deficient in thiamine, and presumably was associated with the presence of this induced deficiency. As in other deficiency studies, the protective action manifested itself mainly by a slowing of the death rate—a delaying rather than an outright protective action. However, as the animals of group 5-V were given some protection to the end of the experiment, it suggests that if the proper conditions could be found, it is possible that the degree of protection might be increased. It is of particular interest that in this same group (5-V), protection was afforded even though oxythiamine was not administered until 4 days after inoculation with the virus.

been the difference in the weight of the mice in the two groups. The animals of oxythiamine groups were not given the analogue until a varying number of days after the low-thiamine group had been started on the diet deficient in the vitamin. During this interval, the mice which later received oxythiamine were growing at a normal rate, while the vitamin-deficient animals were growing subnormally. The growth-rate differential resulted in a difference of about 3 g between the thiamine-deficient and the oxythiamine-fed animals at the time of inoculation, which may

indicate a difference in the degree of deficiency not otherwise recognizable.

Summary. Mice from a genetically controlled colony and under controlled environmental conditions were used to examine the effect of oxythiamine, an inhibitory analogue of thiamine, on resistance against the Lansing strain of poliomyelitis.

A significant degree of protection was induced in all the groups on the oxythiamine as compared with those on the normal diet. This protection was not quite as marked as in the mice on the low-thiamine diet.

16755

Comparison of Penicillin G and A Biosynthetic Penicillin with Regard to Diffusion into Cerebrospinal Fluid.

WILLIAM P. BOGER AND WILLIAM W. WILSON. (Introduced by L. E. Arnow.)

From Philadelphia General Hospital, Philadelphia, Pa.

Under natural conditions *Penicillium notatum* or *Penicillium chrysogenum* produces a mixture of penicillins G, F, dihydro-F, K and X. The predominant form of the penicillin elaborated can be influenced by the addition of precursors to the nutritive media. Similarly, the addition of precursors that do not occur in nature has resulted in the formation of penicillins that do not occur naturally. Such penicillins are formed by reason of the ability of the mold to utilize these precursors and incorporate portions of them into the penicillin molecule.¹ Penicillins produced by the addition of precursors not occurring in nature may be referred to as biosynthetic penicillins. BT penicillin* is such a biosynthetic penicillin and will predominate if the precursor used is one containing the *n*-butylthiomethyl group-ing in available form.

The widespread use of crystalline penicillin G (benzyl penicillin) has established the fact

that many patients show the usual phenomena of drug sensitivity. In addition to the possible advantage of having a penicillin in which a substitution had been made for the benzyl group, it was thought that perhaps such a penicillin would have distinctive properties not possessed by benzyl penicillin. Preliminary observations made in animals suggested that BT penicillin might diffuse into the cerebrospinal fluid more readily than benzyl penicillin.² The investigation here reported was carried out to test this hypothesis.

Materials used. The water-soluble potassium salt of BT penicillin with a potency of 2900 units per mg was employed throughout. The material contained a small amount of penicillin G as a contaminant, for the media upon which the mold was grown during the preparation of this material contained naturally occurring precursors which permitted the formation of penicillin G. The sodium salt of crystalline penicillin G was used for comparison with the BT penicillin.

Patients studied. Eleven patients with central nervous system syphilis were investigated.

¹ Biosynthesis of Penicillins, *Science*, 1947, 104, 503.

* *n*-butylthiomethyl penicillin supplied through the courtesy of Dr. N. P. Sullivan of Eli Lilly & Co.

² Sullivan, N. P., personal communication.

TABLE I.
Plasma Concentrations of Penicillin After Initial Intravenous Injection of 500,000 Units.

Patient	Age, yrs	Color	Sex	Penicillin G*		Penicillin BT†		
				Min. after injection		Min. after injection		
				5	120	5	30	120
BC	57	B	F	63.49‡	.992	34.56	4.32	.270
MW	35	W	F	46.08	.720	23.04	2.16	.180
RR	35	B	M	67.58	.248	34.56	5.74	.270
JS	43	B	M	70.92	1.08	23.04	2.88	.180
JO	52	W	M	46.08	.744	34.56	2.16	.360
McL	38	W	M	31.74	.496	17.18	2.16	.180
IJ	35	B	M	34.56	—§	17.18	2.16	.180
JM	44	W	M	23.81	.996	34.56	2.88	.360
HZ	63	W	M	63.49	1.98	39.94	3.74	.360
MK	41	W	F	31.74	1.49	34.56	6.66	.270
WW	61	W	M	63.49	1.49	64.51	13.31	1.66
Avg				49.36	1.02	32.51	4.37	.388

* Sodium penicillin G in aqueous solution.

† n-butylthiomethyl penicillin in aqueous solution.

‡ Oxford units of penicillin per cc of plasma.

§ Specimen broken.

There was no impairment of general health and no evidence of inflammation of the meninges.

Method of study. Each patient was observed during both periods of the study and each served as his own control. Following a single intravenous injection of 500,000 units of crystalline penicillin G (injection time 5-15 seconds), blood specimens were obtained at 5 and 120 minutes and a specimen of cerebrospinal fluid was obtained from the lumbar subarachnoid space at 120 minutes. These specimens were submitted for penicillin assay by a modification of the Rammelkamp serial dilution method. After an interval of no less than 3 days and no more than 7 days the patient received a single intravenous injection of 500,000 units of BT penicillin and thereafter blood specimens were obtained at 5, 30 and 120 minutes and a cerebrospinal fluid specimen was obtained at the end of 120 minutes. These specimens were likewise assayed for their penicillin content.

Results. The results are presented in Tables I and II. In Table I it may be observed that the average penicillin plasma concentrations resulting from the intravenous injection of 500,000 units of penicillin G were 49.36 units per cc at 5 minutes and 1.02 units per cc at 120 minutes. Following the administration of 500,000 units of BT penicillin, plasma concentrations were 32.51 units per cc

at 5 minutes and 0.388 unit per cc at 120 minutes. The difference between the plasma concentrations observed at 5 minutes following the use of the two penicillins is not significant since the difference represents only a one tube-difference in the Rammelkamp serial dilution method.³ The difference between 1.02 units per cc and 0.388 unit per cc observed at the end of 120 minutes represents a difference of 4 tubes in the Rammelkamp assay and is therefore significant, but the difference is not great enough to postulate any essential difference in rate of elimination of the two penicillins. In Table II it may be observed that, at 120 minutes after the injection of penicillin G, 8 of 11 patients showed assayable quantities of penicillin in the cerebrospinal fluid whereas only 4 of 11 patients showed penicillin in the cerebrospinal fluid following the injection of BT penicillin. These results are barely significant statistically ($p = 0.057$). The difference in actual penicillin concentration observed, that between 0.032 following penicillin G and 0.01 following BT penicillin, is statistically significant ($p = 0.02-0.05$).

Summary. BT penicillin (n-butylthiomethyl penicillin) gave no evidences of toxicity, either immediate or delayed, following the

³ Miller, A. K., and Boger, W. P., *Am. J. Clin. Path.*, 1948, 18, 421.

TABLE II.
Penicillin in Cerebrospinal Fluid Two Hours After Initial Intravenous Injection of 500,000 Units.

Patient	Age, yrs	Color	Sex	Penicillin G*	Penicillin BT†
BC	57	B	F	.045	.0
MW	35	W	F	.0	.0
RR	35	B	M	.031	.0
JS	43	B	M	.045	.0
JO	52	W	M	.0	.045
McL	38	W	M	.0	.0
IJ	35	B	M	.023	.022
JM	44	W	M	.031	.0
HZ	63	W	M	.045	.0
MK	41	W	F	.090	.022
NW	61	W	M	.045	.022
Avg				.032	.010

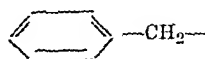
* Sodium penicillin G in aqueous solution.

† n-butylthiomethyl penicillin in aqueous solution.

‡ Oxford units of penicillin per cc of cerebrospinal fluid.

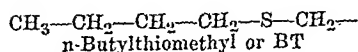
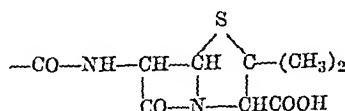
§ Any penicillin concentration less than 0.022 unit per cc has been regarded as zero.

Formula of R



Benzyl or G

Penicillin Nucleus



Structural comparison of the two penicillins employed in this study.

intravenous injection of 500,000 units in 11 patients. From the work here reported it appears that BT penicillin does not diffuse into the cerebrospinal fluid as readily as benzyl penicillin and plasma concentrations resulting from its use are essentially the same as those following the use of benzyl penicillin.

The authors wish to express their indebtedness to Doctors Harvey Bartle, Robert Bookhammer, Raphael Durante, Herbert Freed, and Anthony Frignito for their kindness in allowing us to study patients on their psychiatric services at the Philadelphia General Hospital; to Miss Barbara V. Prey for penicillin assays here reported, and to Mr. Joseph L. Ciminera for the statistical analysis of data.

16756

Effect of Necrosin on Spontaneous Tumors in Mice.

VALY MENKIN.

*From the Agnes Barr Chase Foundation for Cancer Research, Temple University School of Medicine, Philadelphia, Penn.**

The pattern of injury with inflammation has recently been found by the writer to be referable to the liberation of a toxic substance

by injured cells.¹ This substance is located in or at least it is associated with the euglobulin fraction of exudates of dogs and of man. It has been termed necrosin. Recent as yet

* Aided (in part) by a grant from the National Advisory Cancer Council.

¹ Menkin, Valy, *Arch. Path.*, 1943, 30, 269.

unpublished studies have demonstrated a similar substance in injured tissues of invertebrates. The presence of necrosin in canine exudates has been confirmed by Smith and Smith² and by Tanturi.³ Ludford has shown that trypan blue tends to accumulate in some of the cells of the stroma of tumors.⁴ Duran-Reynals has demonstrated that the dye T-1824 and other poorly diffusable dyes localize from the circulating blood in spontaneous tumors of mice, rabbits, and of chickens.⁵ He also showed that the sera of various animals localize from the blood into transplantable and spontaneous tumors of mice.⁵ Duran-Reynals interpreted his findings in the light of a greater permeability on the part of the capillaries of a tumor than under normal circumstances.⁵

In view of these findings it was thought it would be of interest to see whether necrosin, being associated with a protein fraction, would also localize, when injected subcutaneously and at a distance from the tumor, into the tumor substance itself. The effect of the lo-

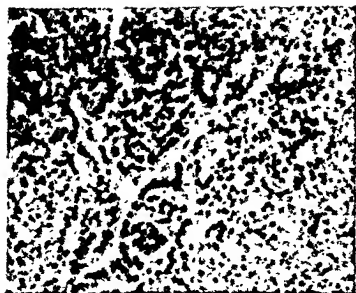


FIG. 1.

An area of the spontaneous tumor which occurs in a strain of Swiss mice. Note the general cellular anaplasia with few areas of adenomatous tendency. $\times 225$.

calization of such a toxic substance into experimental tumors would doubtless also yield valuable information. The experiments to be presently reported represent the first of a series of such observations.

Methods and materials. In a strain of Swiss mice a type of spontaneous tumors, presumably mammary in character, occurs in females.⁶ The tumors may appear almost



FIG. 2.

In such tumor one frequently encounters large vascular sinusoids with marked congestion. These spaces, however, are discrete and appear unruptured. $\times 100$.

² Smith, O. W., and Smith, G. V., *Proc. Soc. Exp. Biol. and Med.*, 1945, 39, 116.

³ Tanturi, C. A., Canefa, J. F., and Banfi, R. F., *Revista "Medicina,"* 1945, 6, 143.

⁴ Ludford, R. J., *Proc. Roy. Soc., ser. B.*, 1929, 104, 493.

⁵ Duran-Reynals, F., *Am. J. Cancer*, 1939, 35, 98.

⁶ Dunn, Thelma B., *Mammary Tumors in Mice* by Members of the Staff of the National Cancer Institute, American Association for the Advancement of Science, Smithsonian Institution Building, Washington 25, D.C., 1945, pp. 13-38.

TABLE I.
Effect of Necrosin on Spontaneous Tumor in Swiss Mice (3 mice were C₃H; 3 mice belonged to dba strain).

No. of mice	No. of mice with frank hemorrhagic necrosis in tumor or in lung metastasis, or necrotic material in tumor	% of mice with frank necrosis in tumor, in metastases, or in both
73 mice inj. with necrosin (1 to 8 inj. subcut.; 0.2 µg to 1748 µg per inj.)	67 (including the C ₃ H and dba mice)	91.8
31 non-inj. mice*	7	22.6

Chemical analysis of one sample of necrosin utilized yielded the following data:

	%
Carbon	41.24
Hydrogen	6.32
Nitrogen	11.58
Sulfur	1.56

* At times very old non-injected mice with very vascular spontaneous tumors display at necropsy variable amounts of necrosis in the tumor, with occasional necrosis likewise occurring in some of the metastases in the lungs. This suggests that canine necrosin administration hastens the time of necrosis to occur in the neoplasms studied.

anywhere in the subcutaneous tissue, but they are more prevalent in the ventral surface of the animal. The tumors are occasionally found in the axilla, in the shoulder, and even in the dorsal surface of the neck. Sometimes these tumors appear in the lower portion of the pelvis. The tumor may show areas of anaplasia interspersed with other areas of adenomatous tendency (Fig. 1). The tumors are characterized by their great vascularity. The presence of large vascular sinusoids is frequently seen (Fig. 2). The tumor is essentially carcinomatous in appearance.

Swiss mice with such tumors, varying in their size, were injected subcutaneously, at a distance from the tumor, with a colloidal solution of necrosin. The amount of necrosin varied from the equivalent of 1748 µg by dry weight to 0.2 µg. The injections, however, were always done in the fluid state in amounts ranging from .05 cc to 0.2 cc. The injections were repeated at intervals of 24 hours or longer. A study of the effect on the tumors was made at 1 hour, 4 hours, 6 hours and 7 hours after the injection of necrosin, and also at the death of the animal. Death occurred usually, though not always, several days after the last injection of necrosin.

Results. The results on over 100 mice are assembled in Table I.† In 73 mice injected with necrosin 91.8% manifested various degrees of necrosis in the tumor substance. Fre-

quently the necrosis was hemorrhagic in character (Fig. 3). The tumor substance became in large part replaced by the free oozing of dark bloody-like material. Only here and there tufts of the original neoplastic tissue remained. Sometimes, although perhaps not as frequently, a mucoid-like yellowish fluid was found instead of the usually relatively firm tumor substance. This was taken to be a type of necrosis. Rarely a caseous-like central necrosis was also apparent in the tumor substance.

In all this work the effect on the metastases was of real interest and perhaps even of greater significance than the effect on the tumor proper. The tumor most frequently metastasizes to lung where the metastases appear, in the gross, as translucent-like foci. Microscopically these foci appear as discrete carcinomatous areas (Fig. 4). The injection of necrosin is accompanied not only by necrosis in the tumor proper, but also by hemorrhagic necrosis in many of the metastatic foci in lung tissue (Fig. 5). The gross appearance of the tumor following necrosin administration is seen in Fig. 6. Microscopically the appearance of the tumor in a treated mouse is, as stated above, exemplified in Fig.

† Since the completion of this manuscript further studies on this subject have been continued. This now involves well over 150 mice in 20 independent series of experiments.

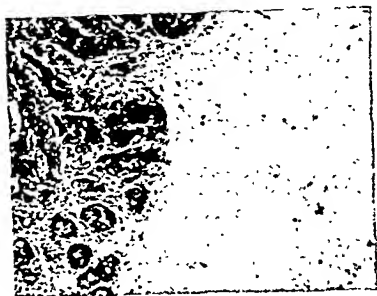


FIG. 3.

This animal received 3 subcutaneous injections of 0.5 cc of necrosin diluted with saline 1:5. Over 2 weeks later the animal died. A large amount of hemorrhagic fluid replaced in large part the tumor substance. $\times 75$.



FIG. 4.

The spontaneous tumor of Swiss mice frequently metastasizes to lung. The illustration represents such metastasis. They appear as discrete carcinomatous areas. $\times 150$.

3. The appearance, in the gross, of the tumor in a mouse treated subcutaneously with another globulin such as the leukocytosis-promoting factor of exudates of dogs⁷ appears in Fig. 7. In brief, the leukocytosis-promoting factor of exudates seems to have no significant effect on the character of the tumor.

Necrosin is found to induce some necrotizing effect on the tumor as early as one hour or so following the subcutaneous injection of the material, in turn administered at a distance from the tumor. An effect can be elicited by the injection of as little as 0.2 μ g of necrosin. The euglobulin fraction of normal canine blood serum usually fails to induce any effect on the tumor or on its metastases. Since necrosin contains a slight amount of pro-

teolytic enzyme,⁸ an attempt was made to determine the effect of papain. The injection of this enzyme seems to induce no significant visible effect. These studies, however, are being continued with other proteolytic enzymes.⁷ Digestion of necrosin for several hours with either crystalline trypsin or papain does not seem to affect significantly the end result on the tumor. Necrosin administered to Swiss mice with spontaneous tumors does not appear to influence the longevity of the treated animal when compared with untreated mice. There may be several factors involved for this lack of effect on the life span. These are at present under investigation. One definite fact is a concomitant severe injury to the liver. This is illustrated in Fig. 8. The injury may be in the form of severe hepatic vacuolation. Sometimes varying degree of cellular infiltration is encountered. The kidneys at times reveal evident injury. A similar effect of necrosin was described in an earlier communication to occur in dogs.¹ Methods of obviating injury to the liver by necrosin are also being studied.

Some degree of necrosis is found in only 22.6% of the tumors of untreated mice (Table I). In such animals, however, the metastatic lesions of the lungs are seldom involved. It is for this reason that a study of the effect of necrosin on the metastatic lesions in the lung may perhaps yield more significant information than its effect on the tumor proper. Only several mice were available which belonged to different strains. These

⁸ Menkin, Valy, *Am. J. Physiol.*, 1946, **147**, 379.

⁷ Since the completion of this manuscript, preliminary studies by the repeated subcutaneous injections of 0.5 mg of crystalline trypsin (Armour) in saline into Swiss mice with spontaneous tumors have yielded hemorrhagic necrosis in only some of the metastatic foci of the lungs. This would perhaps suggest a possible explanation of the action of necrosin, i.e., through its slight degree of proteolytic property. This phase of the work is being studied further.

⁵ These studies are also being done on what has been found to be Strain A mice with apparently the same effect. This will, however, form the subject of a future communication.

⁷ Menkin, Valy, *The Lancet*, May 17, 1947, pp. 660-662.



FIG. 5.
A metastatic focus in the lung of a Swiss mouse having received 3 subcutaneous injections of 0.5 cc of necrosin diluted with saline 1:5. Over 2 weeks later the animal succumbed. The metastatic area manifests considerable hemorrhagic necrosis. $\times 85$.



FIG. 6.

Gross appearance of a spontaneous tumor in a Swiss mouse in turn injected subcutaneously twice with .05 cc of necrosin diluted with saline (1:5). The amount of necrosin was equivalent to 10 mg per injection. The animal was sacrificed one day following the second injection. Note the marked degree of hemorrhagic necrosis.



FIG. 7.

Gross appearance of a spontaneous tumor in a Swiss mouse following the subcutaneous injection of 0.1 cc of canine leukocytosis-promoting factor (α 1 and α 2 globulins of exudates). The mouse died 5 days after administration of the globulin material. There is no evidence of any necrosis in the tumor.

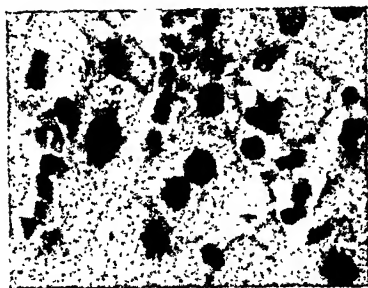


FIG. 8.

Appearance of the liver in a mouse injected subcutaneously twice with .05 cc of necrosin. The mouse succumbed 4 days following the second injection. Note the severe degree of injury to the liver in the form of marked vacuolation within the hepatic cells. $\times 560$.

were C H and dba mice.⁵ Repeated subcutaneous injections of necrosin were³ likewise followed by some degree of tumor necrosis with concomitant necrotizing effect on some of the lung metastasis. In these animals, however, many more injections of this toxic material were necessary, and the effect was generally not as pronounced as on the Swiss strain of mice. The extensive initial vascularity of the tumor has probably something to do with the ultimate effect of necrosin administration. Finally, in view of Shear's⁶ recent work on bacterial polysaccharides necrosin was tested for the Molisch reaction, and it was found on a sample utilized to be negative. Culturing necrosin for bacteria in broth yielded negative results within eleven hours,

⁵ Shear, M. J., *J. National Cancer Inst.*, 1944, 4, 461.

and negative growth on agar slants within 24 hours. The necrosin samples employed ranged from fresh material (only several hours old) to material prepared over 2 years previously and preserved in a refrigerator. The effects obtained were identical. Finally, the subcutaneous injections of 0.2 cc of a slightly acid exudate (pH 6.9 to 7.0), in which presumably necrosin was present, induced hemorrhagic necrosis in the tumor and in some of the metastatic foci of Swiss mice.^{||}

Conclusions. Necrosin is a substance associated with the euglobulin fraction of usually acid inflammatory exudates. Its liberation by injured cells offers a reasonable explanation for the basic pattern of injury in inflammation.

The subcutaneous injections of canine necrosin, at a distance from the site of spontaneous tumors, in a strain of Swiss mice produce, after varying intervals, hemorrhagic or other types of necrosis in the tumor substance.

A necrotizing effect is also seen to occur in some of the metastatic lesions encountered in the lung.

Necrosin injections in these mice induce severe injury to the liver.

None of the effects on the tumor or on its metastasis are usually encountered with the use of the euglobulin fraction of canine blood serum, the leukocytosis-promoting fraction of canine exudates, or with one of the proteolytic enzymes adequately studied, namely papain.

^{||} My thanks are due to Mrs. Ruth Lewin for technical aid in the course of this study.

16757

Effect of Adrenocorticotrophic Hormone on Anaphylaxis in the Guinea Pig.

JACQUES LEGER,* W. LEITH AND BRAM ROSE. (Introduced by J. S. L. Browne.)

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Adrenal cortical hormones are known to cause dissolution of lymphoid tissue,^{1,2} and it

* Fellow National Research Council, Canada.

¹ Dougherty, T. F., and White, A., *Proc. Soc. Exp. Biol. and Med.*, 1943, 33, 132.

has recently been shown that in sensitized animals, the rate of lymphocyte disintegration is paralleled by an increase of circulating antibodies.³⁻⁶ This phenomenon may be elici-

² Selye, H., *J. Clin. Endocrinol.*, 1946, 6, 117.

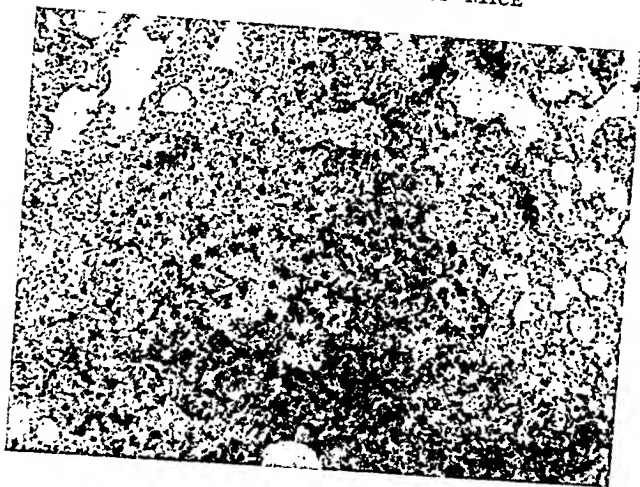


Fig. 5.
A metastatic focus in the lung of a Swiss mouse having received 3 subcutaneous injections of 0.5 cc of necrosin diluted with saline 1:5. Over 2 weeks later the animal succumbed. The metastatic area manifests considerable hemorrhagic necrosis. $\times 85$.



Fig. 6.
Gross appearance of a spontaneous tumor in a Swiss mouse in turn injected subcutaneously twice with .05 cc of necrosin diluted with saline (1:5). The amount of necrosin was equivalent to 10 mg per injection. The animal was sacrificed one day following the second injection. Note the marked degree of hemorrhagic necrosis.



Fig. 7.
Gross appearance of a spontaneous tumor in a Swiss mouse following the subcutaneous injection of 0.1 cc of canine leukocytosis-promoting factor (α 1 and α 2 globulins of exudates). The mouse died 5 days after administration of the globulin material. There is no evidence of any necrosis in the tumor.

mg A.C.T.H. for mice, 60 to 80 days of age and 10 mg of A.C.T.H. for 4-month-old rabbits, it was considered that weight for weight $3\frac{1}{2}$ to 4 mg of A.C.T.H. would be a suitable dose for the guinea pigs used. The animals were carefully observed for the appearance of anaphylactic shock which was graded as follows: mild shock, severe shock, and death.

Results. The results of this experiment are summarized in Table I. The animals in both groups showed a similar response to the shocking dose of the antigen. Group I, the control group, all showed varying degrees of shock, which was fatal in 12 of 17 animals, with 3 showing severe shock and 2 showing symptoms of mild shock. Group II, the experimental group, also showed 100% reactivity, there being 12 instances of fatal shock, 3 cases of severe shock, and mild shock in 3

animals. The time of death occurred at approximately the same time interval following the shocking dose in both groups of animals. Therefore, one may conclude that A.C.T.H. in the dose administered, and under these experimental conditions, does not seem to have any effect on anaphylactic shock as elicited in guinea pigs by the procedure outlined above.

Summary. The administration of adrenocorticotrophic extract to sensitized guinea pigs prior to injection of a shock dose of antigen failed to influence the course of anaphylactic shock in a group of 18 animals.

We wish to thank Dr. J. F. Mote of Armour Laboratories for the preparation of A.C.T.H., and Mr. Feith for technical assistance. The horse serum was kindly supplied by Prof. L. Forte of the Université de Montreal.

16758

In vitro Studies of Aureomycin, a New Antibiotic Agent.*

ELEANOR A. BLISS AND CAROLINE A. CHANDLER. (Introduced by P. H. Long.)

From the Departments of Preventive Medicine and Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, Md.

Samples of a new antibiotic, designated as A377 and later named aureomycin, were received from the Lederle Laboratories Division of the American Cyanamid Company, in November 1947. Since preliminary trials showed that the product had considerable activity *in vitro* against both a gram negative bacillus and a gram positive coccus, it was decided to test it further.

Description. According to information received from the Lederle Laboratories, aureomycin is derived from a strain of *Streptomyces aureofaciens*. The partially purified hydrochloride which was sent us is a bright yellow, fluffy, crystalline material. A 10%

solution can be prepared in distilled water but the solubility is lowered by the presence of sodium chloride. The pH of a 0.1% aqueous solution is approximately 4. Dilute broth solutions pass through Berkefeld N and Seitz filters with but little loss of activity.

Range of Action. The least amount of aureomycin which prevents visible growth for 24 hours was determined for a variety of gram positive and gram negative bacteria. Polymyxin¹ and penicillin were used as standards of comparison in parallel tests. Serial 2-fold dilutions of the drugs in Difco Heart Infusion Broth, with 0.075% dextrose added, were inoculated with equal volumes of 18 hour old cultures so diluted as to result in approximately 200,000 organisms per cc. The diluent for the gram negative bacilli was

* These investigations were supported by grants from Abbott Laboratories, Eli Lilly and Company, Lederle Laboratories Division of the American Cyanamid Company, Parke, Davis and Company, and The Upjohn Company.

¹ Stansly, P. G., Shepherd, R. G., and White, H. J., *Bull. Johns Hopkins Hosp.*, 1947, **81**, 43.

TABLE I.
Influence of A.C.T.H. on Anaphylactic Shock.

Animals	Sensitizing dose, cc	Incubation period, days	Control group.		Symptoms	% shock
			Shocking dose, cc	A.C.T.H. mg		
1	.25	12	0.5		Severe	
2	.25	12	0.5		Fatal	
3	.25	12	1.0		"	
4	.25	12	1.0		"	
5	.25	12	1.0		"	12 died
6	.25	12	1.0		"	
7	.25	12	1.0		"	
8	.25	17	0.5		"	
9	.25	17	0.5		Mild	100% shock
10	.25	17	0.5		Fatal	
11	.25	17	0.5		Severe	
12	.25	17	0.5		Fatal	
13	.25	17	0.5		Severe	
14	.25	17	0.5		Fatal	
15	.25	17	0.5		Mild	
16	.25	17	0.5		Fatal	
17	.25	17	0.5		"	
Experimental group.						
1	.25	13	1.0	4	Fatal	
2	.25	13	0.5	4	"	
3	.25	13	1.0	4	Severe	
4	.25	13	1.0	4	Fatal	
5	.25	13	1.0	4	"	12 died
6	.25	13	1.0	4	Mild	
7	.25	13	1.0	4	Fatal	
8	.25	13	0.5	3.5	"	
9	.25	17	0.5	3.5	Severe	100% shock
10	.25	17	0.5	3.5	Fatal	
11	.25	17	0.5	3.5	Mild	
12	.25	17	0.5	3.5	Fatal	
13	.25	17	0.5	3.5	Severe	
14	.25	17	0.5	3.5	Mild	
15	.25	17	0.5	3.5	Fatal	
16	.25	17	0.5	3.5	"	
17	.25	17	0.5	3.5	"	
18	.25	17	0.5	3.5	"	

Mild shock denotes symptoms of dyspnea, sneezing, diarrhea and restlessness.
Severe shock denotes symptoms of dyspnea going on to convulsions and unconsciousness.
Fatal refers to death.

ted by administration of adrenal cortical hormones, or by stimulation of the adrenal cortex by means of A.C.T.H. (adrenocorticotrophic hormone). The following investigation was carried out in order to study the effect of A.C.T.H. on anaphylactic shock in the guinea pig.

Methods. Thirty-five male and female

3 Dougherty, T. F., Chase, J. H., and White, A., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 295.

4 Dougherty, F. F., White, A., and Chase, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 28.

5 Dougherty, T. F., Chase, J. H., and White, A., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 135.

6 Dougherty, T. F., and White, A., *J. Lab. and Clin. Med.*, 1947, **32**, 584.

guinea pigs weighing between 200 - 400 g were injected intraperitoneally with 0.25 cc of undiluted horse serum. These animals were submitted to a re-injection of $\frac{1}{2}$ to 1 cc of the same antigen after an incubation period varying between 12 to 17 days. The readministration was performed via the jugular vein, which had been exposed by dissection in the unanaesthetized animal. The animals were divided into two groups, Group I comprising 17 control guinea pigs, and Group II comprising the remaining 18 animals. The animals of Group II were given $3\frac{1}{2}$ to 4 mg of A.C.T.H. (s.c.) 6 to 8 hours prior to the shocking dose of the antigen. Since Dougherty and his colleagues used 1

mg A.C.T.H. for mice, 60 to 80 days of age and 10 mg of A.C.T.H. for 4-month-old rabbits, it was considered that weight for weight $3\frac{1}{2}$ to 4 mg of A.C.T.H. would be a suitable dose for the guinea pigs used. The animals were carefully observed for the appearance of anaphylactic shock which was graded as follows: mild shock, severe shock, and death.

Results. The results of this experiment are summarized in Table I. The animals in both groups showed a similar response to the shocking dose of the antigen. Group I, the control group, all showed varying degrees of shock, which was fatal in 12 of 17 animals, with 3 showing severe shock and 2 showing symptoms of mild shock. Group II, the experimental group, also showed 100% reactivity, there being 12 instances of fatal shock, 3 cases of severe shock, and mild shock in 3

animals. The time of death occurred at approximately the same time interval following the shocking dose in both groups of animals. Therefore, one may conclude that A.C.T.H. in the dose administered, and under these experimental conditions, does not seem to have any effect on anaphylactic shock as elicited in guinea pigs by the procedure outlined above.

Summary. The administration of adrenocorticotrophic extract to sensitized guinea pigs prior to injection of a shock dose of antigen failed to influence the course of anaphylactic shock in a group of 18 animals.

We wish to thank Dr. J. F. Mote of Armour Laboratories for the preparation of A.C.T.H., and Mr. Feith for technical assistance. The horse serum was kindly supplied by Prof. L. Forte of the Université de Montreal.

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In vitro Studies of Aureomycin, a New Antibiotic Agent.*

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Samples of a new antibiotic, designated as A377 and later named aureomycin, were received from the Lederle Laboratories Division of the American Cyanamid Company, in November 1947. Since preliminary trials showed that the product had considerable activity *in vitro* against both a gram negative bacillus and a gram positive coccus, it was decided to test it further.

Description. According to information received from the Lederle Laboratories, aureomycin is derived from a strain of *Streptomyces aureofaciens*. The partially purified hydrochloride which was sent us is a bright yellow, fluffy, crystalline material. A 10%

solution can be prepared in distilled water but the solubility is lowered by the presence of sodium chloride. The pH of a 0.1% aqueous solution is approximately 4. Dilute broth solutions pass through Berkefeld N and Seitz filters with but little loss of activity.

Range of Action. The least amount of aureomycin which prevents visible growth for 24 hours was determined for a variety of gram positive and gram negative bacteria. Polymyxin¹ and penicillin were used as standards of comparison in parallel tests. Serial 2-fold dilutions of the drugs in Difco Heart Infusion Broth, with 0.075% dextrose added, were inoculated with equal volumes of 18 hour old cultures so diluted as to result in approximately 200,000 organisms per cc. The diluent for the gram negative bacilli was

* These investigations were supported by grants from Abbott Laboratories, Eli Lilly and Company, Lederle Laboratories Division of the American Cyanamid Company, Parke, Davis and Company, and The Upjohn Company.

¹ Stansly, P. G., Shepherd, R. G., and White, H. J., *Bull. Johns Hopkins Hosp.*, 1947, **81**, 43.

TABLE I.

Comparative Activity of Aureomycin (A377), Penicillin, and Polymyxin (B71) for Various Bacteria.

Bacterium	Group or type	Medium	Inoculum, cc	Minimal inhibitory conc., γ /cc (24 hr)			A/P ratio
				A377	Peni.	B71	
<i>Str. hemolyticus</i>	A-C203	Blood HIB	$.5 \times 10^{-3}$.3125	.006		50
" "	D-Zymog*	"	"	1.25	2.5	> (20)	.5
" "	F-For	"	"	.625	.05		12.5
" "	G-Dog	"	"	1.25	.0125		100
<i>D. pneumoniae</i>	I SV1	"	"	.3125	.025		12.5
" "	I Bailey	"	"	.3125	.0125		25
" "	II	"	$\times 10^{-4}$.3125			
" "	III	"	"	.156			
<i>Str. faecalis</i>	D-Tarr*	"	$\times 10^{-3}$	1.25	>1		< 1
<i>Staphylococcus</i> (all are aureus)	Barlow	"	"	.625	.025		25
	Zeut	"	"	.62	.05		12.5
	Zorn	"	"	.62	.05		12.5
<i>B. subtilis</i>	Lederle	"	"	.08			
" "	FDA	"	"	2.5			
" "	"	Plain HIB	"	2.5			
<i>E. coli</i>	4 SI	"	$\times 10^{-4}$	5.0		.156	32
" "	9	"	"	5.0		.156	32
<i>A. aerogenes</i>	7	"	"	5.0		.3125	16
" "	8	"	"	5.0		.625	8
<i>K. pneumoniae</i>	Lederle	"	"	1.25		.156	8
" "	Cephus	"	"	5.0		.625	8
<i>Ps. aeruginosa</i>	16	"	"	>20		2.5	> 8
" "	Calloway	"	"	>20		2.5	> 8
<i>Proteus</i>	17	"	"	>20		>20	
" "	18	"	"	>20		>20	
<i>H. influenzae</i>	b-Pittman	Fildes-HIB	—	2.0		.35	6

* Four additional strains of Group D streptococci (α and β) were tested. All were inhibited by 1.25 γ /cc A377 and were less sensitive to penicillin.

heart infusion broth, that for the cocci contained 4% of washed rabbit red cells. The tests were read after 18 hours incubation at 37°C.

The results are shown in Table I. It is apparent that, in the test tube and with the inocula used here, aureomycin is less effective than polymyxin and penicillin against the gram negative bacilli and the gram positive cocci, respectively. The only exception noted was in the case of the Group D streptococci. Both α and β strains of these organisms were more susceptible to aureomycin than to penicillin. In the concentrations tested here, neither aureomycin nor polymyxin checked the growth of *Proteus*.

Comparison was also made with streptomycin in a few instances, with the results shown in Table II. Aureomycin was less effective than streptomycin in suppressing, for 18 hours, the growth of *E. coli* and *K. pneumoniae*. The strain of staphylococcus which was used in this test was more susceptible to the new agent than to streptomycin, while the two agents were equally effective against *H. influenzae* b.

Bacteriostatic vs. Bactericidal Activity. In the course of the above tests it was noted that on further incubation the end-points for aureomycin moved up day by day. Moreover, subcultures on blood agar plates made after the first day of incubation showed the presence of

TABLE II.
Comparative Activity of Aureomycin (A377) and Streptomycin for Various Bacteria, at the
End of 24 and 48 Hours Incubation.

Organism	Drug	End-point— γ /cc			A/S ratio 24 hr
		Turbidity 24 hr	Turbidity 48 hr	Subculture 24 hr	
<i>E. coli</i>	A377	10.0	20.0	>30.0	2.0
	Streptomycin	5.0	5.0	5.0	
<i>Staph. aureus</i>	A377	1.0	10.0	>20.0	0.1
	Streptomycin	10.0	10.0	10.0	
<i>K. pneumoniae</i>	A377	2.5	10.0	15.0	2.0
	Streptomycin	1.25	1.25	1.25	
<i>H. influenzae</i>	A377	2.0	2.0	2.0	0.67
	Streptomycin	3.0	3.0	3.0	

Note: Medium—Heart infusion broth, except in case of *H. influenzae* where Fildes broth was used.

TABLE III.
Growth Rate of *E. coli* and Beta Streptococcus in the Presence of Aureomycin (A377),
Polymyxin (B71), and Penicillin.
(a) *E. coli*—Heart Infusion Broth.

Time of plating Hr after inoculation	Broth control	A377 20 γ /cc* Organisms per cc	B71 0.2 γ /cc†
	$\times 1000$	$\times 1000$	$\times 1$
0	220	230	310,000
1	430	90	20
3	16,000	35	<10
6	300,000	12	<2
24	5,000,000	13	<2
72	—	++++	—

(b) Beta Streptococcus C203—Heart Infusion Broth + Blood.

	Broth control		A377 1.25 γ /cc*		Penicillin 0.025 γ /cc*	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
			Organisms per cc $\times 1000$			
0	140	275	40	305	120	290
1	200	545	90	95	40	30
3	4000	—	42	—	5	—
4	—	19,000	—	12	—	.3
5	115,000	—	23	—	1.8	—
24	550,000	—	6	—	.03	—
48	—	++++	—	++++	—	0
72	—	—	++++	—	0	—

* $\frac{1}{4}$ times minimal inhibitory concentration for test organism.

† Maximum turbidity.

‡ This test was run on another occasion; 0.2 γ /cc polymyxin is only slightly more than the minimal inhibitory concentration for *E. coli*.

viable organisms in tubes containing 20 times the amount of antibiotic which had prevented visible growth.

These observations, illustrated in Table II, indicated that aureomycin is bacteriostatic rather than bactericidal in its action. This

point has been confirmed by studies of the rate of growth of *E. coli* and the C203 strain of hemolytic streptococcus in the presence of $\frac{1}{4}$ times the inhibitory concentration of the agent. In the case of C203 comparison was made with penicillin. Ten cc volumes of the

TABLE IV.
Deterioration of Aureomycin (A377) after Incubation Alone and in Presence of Test Organism.

Duration of preliminary incubation, hr	Visible end-point γ /cc		
	24 hr reading	48 hr reading	72 hr reading
*	0	1.25	5.0
	24	10	20
	48	20	>20
†	0	1.25	2.5
	24	2.5	10
	48	5	>20

* 40 γ /cc A377 in heart infusion broth after 0 hr, 24 hr, 48 hr incubation—titrated against test organism (*Staph. aureus*).

† Supernatant of 40 γ /cc A377 after incubation with live *Staph. aureus* at 0 hr, 24 hr, 48 hr—titrated against test organism (*Staph. aureus*).

solutions were inoculated with 0.1 cc of 1:100 dilutions of the cultures. Plates were poured at intervals. The results, shown in Table III a and b, indicate that although aureomycin brings about a progressive decline in the bacterial population for the first 24 hours of exposure, the organisms eventually recover and reach maximum growth. In contrast, penicillin caused an immediate decline in the bacterial count which terminated in the sterilization of the cultures. Polymyxin, in a concentration only slightly above the minimal inhibitory concentration, has been observed, in other studies,² to exert an extremely rapid bactericidal effect upon *E. coli*. Aureomycin is clearly less decisive in its action than are penicillin and polymyxin.

It is not known at present whether the eventual outgrowth, in the case of aureomycin, is the result of the survival of resistant organisms or is due to the deterioration of the drug. As shown in Table IV, when aureomycin was titrated in the usual manner after incubation at 37°C in broth alone or in the presence of the test organism for 24 and 48 hours, there was progressive and rapid inactivation.

Confirmatory evidence of this phenomenon was obtained in another set of experiments in which *K. pneumoniae* was the test organism. Three sets of tubes, each containing 2-fold dilutions of aureomycin from 0.625 γ /cc through 20 γ /cc were inoculated in the usual manner. After 24 hours incubation, growth

was found to have been inhibited by 2.5 γ /cc per tube of drug in all 3 sets of tubes. The first set (a) was allowed to incubate without further addition of drug through 72 hours. Fresh drug, to give an additional 2.5 γ /cc per tube, was added to the second set (b) at 24 hours, and to the third set (c) at 24 and 48 hours. As shown in Fig. 1, the inhibitory concentration in (a) was 20 γ /cc at 48 hours and more than 20 γ /cc at 72 hours. Growth in (b) was inhibited by 2.5 γ /cc for 48 hours and then titrated to more than 20 γ /cc at 72 hours. In (c), however, with drug replenished at 24 and 48 hours, the original inhibitory titre of 2.5 γ /cc was maintained throughout the 72 hour observation period. With no further addition of drug to set (c) at 72 hours, growth occurred through 20 γ /cc at 96 hours.

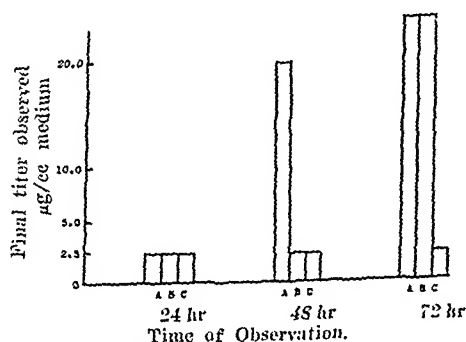


FIG. 1.
Effect of adding increments of fresh aureomycin.

TABLE V.
Effect of Normal Human Serum on the End-points of Titrations of Aureomycin.

Effect of Normal Human Serum on the End-points of Precipitation								
Bacterium	Medium	Serum concentration, %						
		50	25	12½	6¼	3½	1½	0
		Minimal inhibitory conc. of A377, γ/cc						
<i>E. coli</i>	S1G1	25	12.5	6.25	6.25	5	1.56	1.25
	H1B	80	15	10				4
Strept. C203	H1B + Blood	6.25	1.25	0.625	0.312	0.312	0.125	0.125

TABLE VI.
Instability of Aureomycin to Heat. Minimal Inhibitory Concentrations for *E. coli* after Various Exposures.

Duration of exposure	Temperature, centigrade			
	10	24	37	56
		Minimal inhibitory conc., γ/cc		
0 min.			6.25	6.25
25 "		6.25		
40 "				6.25
1 hr				12.5
2½ "		6.25		
4 "				25.0
5 "		6.25		
18 "	6.25		20	
24 "	6.25	12.5		

Effect of Environmental Factors on Titration. Medium. In contrast to that of the sulfonamides, the activity of aureomycin for *E. coli* is but little better in the synthetic medium, S1G1,³ than it is in heart infusion broth. The addition of serum, however, to either medium has a marked effect upon the titration end point. As shown in Table V, the end points vary directly with the concentration of serum. In this respect aureomycin resembles penicillin. The effect, however, is very much greater on the new antibiotic, 50% serum causing a 20- to 50-fold raising of the end-point of aureomycin and only a 4-fold increase in the case of penicillin.

Temperature. Aureomycin showed progressive loss of activity when held, in broth solutions, at higher than refrigerator temperatures. As shown in Table VI, at room temperature there was no change for 5 hours, but by 24 hours half the activity had been lost. Storage at 37°C for 18 hours before use reduced activity by 60%. At 56°C the rate of deterioration increased; 50% of the activity

was lost in 1 hour, 75% in 4 hours.

Relation of Size of Inoculum to Minimal Inhibitory Concentration. Seven sets of serial 2-fold dilutions of aureomycin, containing 0 to 50 γ/cc, were set up in Wassermann tubes. Each set was inoculated with one of a series of 10-fold dilutions, in blood broth, of an 18 hour culture of C203. The tests were incubated at 37°C for 18 hours. It was found that the end-point depends, to a certain extent, upon the number of organisms inoculated. Inocula of 10⁻⁵ to 10⁻⁷ cc of culture were inhibited by 0.156 γ/cc aureomycin, while 0.312 γ/cc was required to delay the development of 10⁻² to 10⁻⁴ cc. The end-point obtained with 1/10 cc of culture could not be determined because there was sufficient streptolysin present in the inoculum to hemolyze the red blood cells.

Summary. A new antibiotic, aureomycin, is effective *in vitro* against both gram positive and gram negative bacteria. Although its activity is of a lower order than that of penicillin for gram positive cocci or polymyxin for gram negative bacilli, it is effective against both classes of bacteria in concentrations close

³ Bliss, E. A., and Long, P. H., *Bull. Johns Hopkins Hosp.*, 1941, 69, 14.

to those required of streptomycin. Inhibition at such concentrations, however, is fleeting in the case of aureomycin, much larger amounts being required for permanent suppression of growth. The new agent is bacteriostatic rather than bactericidal in its effect. Its antibacterial action is greatly diminished in the presence of serum, *in vitro*, and deterioration at room temperature is marked.

Differences in the size of the inoculum have a moderate effect upon the minimal inhibitory concentration.

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Further Studies in Experimental Allergic Encephalitis in the Guinea Pig.

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It has been indicated^{1,2} that an encephalitic process, presumably allergic in nature, results in guinea pigs inoculated with rabbit-brain tissue and "adjuvants". A brief report is herein given of additional experiments with brain-tissue inocula derived from various animal species, as well as on the effect of progressive dilutions of brain-tissue inocula, attempts at purifying the antigen, effect of intracerebral re-injection of the antigen, and on the development of antibrain antibodies in animals inoculated either subcutaneously alone, or subcutaneously and then followed by an intracerebral inoculation of the brain suspension.

1. *Inoculation of brain tissue from various animal species.* The technic followed was the same as reported previously² except that brain material from 5 different species was used for the subcutaneous inoculation. Paralysis of inoculated guinea pigs was observed usually 21 or more days after the injection. Death followed from 1 to 15 days after onset of paralysis and occasionally occurred in animals which had shown no neurological manifestations. The results obtained (Table I) do not lend themselves to a satisfactory comparison

because of the low and unequal number of animals used in each group. In general, the percentage of animals which succumbed was proportionately similar in each group regardless of the source of the brain tissue used in the inoculum.

Lesions in the central nervous system (CNS), of the type already described,² were observed in all animals. In general no conspicuous differences were noted in the lesions which could be ascribed to the use of heterologous antigens, but in some guinea pigs more extensive lesions were obtained when guinea pig brain suspensions were used as inoculum.

2. *Effect of progressive dilutions of brain tissue inoculum.* Phenolized rabies vaccines prepared from calf and rabbit brain tissue, respectively, were used as source material for the inoculum. Starting with the 1:20 dilution of brain emulsion, serial twofold dilutions were made in saline, incorporated into adjuvants and injected into guinea pigs. For the sake of brevity, the results of the experiment (Table II) were grouped irrespective of the brain tissue used as inoculum. For comparison are included data summarizing the results of various experiments in which guinea pigs were inoculated with a suspension of normal rabbit brain tissue in 1:10 dilution. Up to and including the 1:80 dilution, there appeared to be no material difference in the

¹ Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, **57**, 179.

² Jervis, G. A., and Koprowski, H., *J. Neuro-path. and Exp. Neurol.*, 1948, **7**, 309.

TABLE I.

Results Obtained in Guinea Pigs Injected with Brain-tissue Inocula Derived from Various Animal Species.

Brain tissue used	Guinea pigs inoculated, No.	Guinea pigs			
		Paralyzed		Died	
		No.	%	No.	%
Rabbit	94	45	48	39	41
Guinea pig	20	11	55	10	50
Human	20	7	35	11	55
Calf	19	9	47	7	37
Swine	9	6	66	6	66
Total	162	78	48	73	45

TABLE II.

Results Obtained in Guinea Pigs Inoculated with Various Dilutions of Rabies Vaccines.

Dilution of brain tissue	Guinea pigs inoculated, No.	Guinea pigs			
		Paralyzed		Died	
		No.	%	No.	%
1:20	19	9	47	6	32
1:40	15	10	66	10	66
1:80	19	10	52	13	68
1:160	20	4	20	5	25
1:320	19	1	5	2	10
1:10*	162	78	48	73	45

* Normal rabbit-brain tissue used as inoculum.

number of paralyzed or dead animals, and the dilution used. However, a definite decline in the incidence of paralysis or death was observed among the animals inoculated with the 1:160 and 1:320 dilutions.

3. *Attempt at purification of brain antigen.* Precipitation with methanol³ failed either to enhance or to inhibit the antigenic property of the human brain substance and further fractionation was done by Klenk's technic.⁴ The methanol-chloroform extracted fraction, which contains cerebrosides and sphingomyelin, caused paralysis in 2 animals of 10, while 3 of 10 animals showed paralysis following the injection of the protein fraction remaining after extraction of all lipoids. The pathological picture differed in no way from that obtained with the whole brain tissue.

The results obtained with the lipid fraction conform to expectation since it has been

shown that the substance responsible for the allergic reaction is present in the myelin sheaths^{5,6} which have a high cerebroside content. Alvord⁶ has recently reported that the phosphatide fraction obtained with Bloor's technic is also effective in producing allergic encephalitis. Apparently the antigenic property is shared by more than one substance present in the white matter of the CNS. It should be noted, however, that the fractions used are not chemically pure substances but only mixtures of cerebrosides and sphingomyelins, or of lecithins and cephalins, containing many impurities. The purification and identification of the antigen awaits further study and elucidation.

4. *Intracerebral re-inoculation of the antigen.* Five to 7 weeks after the primary subcutaneous injection of brain antigen combined with adjuvants, guinea pigs which had survived were given intracerebrally 0.1 ml of

³ Koprowski, H., Black, J., and Cox, H. R., *Proc. 17th Internat. Congr. Microbiol.*, Copenhagen, 1947, in press.

⁴ Klenk, E., *Hoppe-Seyler Z., f. physiol. Chem.*, 1939, 262, 128.

⁵ Kabat, A. E., Wolf, A., and Bezer, E. A., *J. Exp. Med.*, 1947, 85, 117.

⁶ Alvord, E. C., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1948, 67, 459.

TABLE III.
Results of Intracerebral Inoculation of Guinea Pigs Previously Sensitized by Subcutaneous Injection of Homologous or Heterologous Antigen.

Brain tissue inoculated subcut.	Brain tissue used in subsequent intracerebral inoculation*			
	Rabbit	Guinea pig	Human	Calf
Rabbit	17/19	1/2		
Guinea pig	0/3	1/6		
Human			9/9	
Calf	0/3	0/3		3/3

* Denominator denotes the number of guinea pigs inoculated, numerator the number which died.

10% suspension of brain tissue in saline. With one exception, a high mortality ratio was obtained (Table III) in those groups which had received intracerebral inoculation of the same species-brain tissue suspension to which they had been previously sensitized. Death of the animals, occurring 3 to 15 days after the intracerebral inoculation, was usually preceded by signs of tremors, tonic spasms of the neck, paralysis, and occasionally by convulsions. The exception, mentioned above, occurred in the group of 6 animals which were injected both subcutaneously and intracerebrally with guinea pig brain. In this group only 1 of 6 animals died and its death may have been accidental since no marked pathologic changes were noted on examination of the brain tissues (see below). In the remaining groups of guinea pigs which had received intracerebrally brain tissue suspensions of animals other than from those to which they had been previously sensitized by subcutaneous inoculation, only 1 of 11 died.

Histopathological examinations² were made of the brain and spinal cord tissues of all guinea pigs that died, and of those which survived and had been sacrificed approximately 30 days after inoculation. Conspicuous changes were observed at the site of injection. In animals which had died within a few days after the inoculation, the local lesion consisted of a large necrotic hemorrhagic area. Only tissue-debris was seen at the center, while at the periphery large numbers of granular compound corpuscles and rich perivascular infiltrations of hematogenous elements were observed. In animals which died after the first week, hematogenous ele-

ments were scanty and infiltrations with compound granular elements predominated so that the local lesion was mainly one of demyelination with central necrosis. Furthermore, throughout the CNS, numerous disseminated areas of demyelination and perivascular infiltration of the type previously described² were observed. The fact that the animals had shown no conspicuous neurological signs before the intracerebral injection suggests that at least some of these lesions were of recent appearance and followed the intracerebral administration of antigen, and not older lesions occasioned by the subcutaneous injection. The pathological changes were most striking in those animals which received intracerebrally the same species-antigen to which they had been sensitized, and much less severe in those which had received antigen derived from two different species. It was, however, a difference in degree rather than in quality. The 6 animals (Table III) which were injected both subcutaneously and intracerebrally with guinea pig brain were an exception, since the local pathological lesion was mild.

Ten control animals which received intracerebral injections of brain tissue, without previous sensitization, showed no clinical signs and only minimal pathological lesions.

The focal necrotic lesions of the brain observed in these experiments bore a resemblance to those reported in experimental Arthus-phenomenon in the brain of rabbit,⁷ guinea pig,⁸ and monkey.⁹ That the lesion

⁷ Davidoff, L. M., Seegal, B. C., and Seegal, D., *J. Exp. Med.*, 1932, 55, 163.

⁸ Alexander, L., and Campbell, A. C. P., *Am. J. Path.*, 1937, 13, 229.

was more marked when the intracerebral inoculum was from the same species as that injected subcutaneously, conforms to the hypothesis that the pathological changes are anaphylactic in nature. However, the process seems to be of uncommon intensity in these experiments as evidenced by the high mortality ratio. It is interesting to note that Freund¹ also observed high mortality after re-inoculation of the brain substance intracutaneously into a sensitized animal. These findings may offer some indication as to the possible allergic nature of the encephalomyelitic process.

5. *Determination of antibrain antibodies in animals.* The presence of antibrain antibodies in the blood serum of guinea pigs was determined by complement-fixation tests against three antigens: human, normal guinea pig and rabbit brain which were prepared according to the technic of Casals and Palacios.¹⁰ The technic of the test itself followed the one generally employed in this laboratory¹¹ except that 1.5 units of guinea-pig complement was used. Determinations were performed (a) in animals injected subcutaneously as described under items 1 and 3, and (b) in animals first injected subcutaneously and then intracerebrally as described under item 4.

(a) Complement-fixing antibodies against rabbit brain antigen were first noted on the 7th day after the subcutaneous injection of rabbit-brain suspension combined with adjuvants. On the 11th day, antibodies against brain antigens of all 3 species appeared, and the titer rose rapidly reaching a peak between the 16th and 21st day, followed by a gradual decline. In 2 of the 4 animals examined, antibodies were still present on the 193rd day after injection. With the rabbit-brain inoculum, the titer of antibodies in serum was

always higher when tested against rabbit-brain antigen than against guinea pig or human antigen. In further experiments, it was noted that brain-specific antibodies appear in all injected animals irrespective of the species-source of the brain tissue inoculum. The only significant exception was the guinea pig brain antigen with which, while it induced marked incidence of paralysis in the inoculated animals (Table I), no antibrain antibodies were demonstrated. In general, no correlation was observed between the titer of antibrain antibodies and the clinical picture presented. Following the injection of rabbit-brain tissue, incorporated in Bayol F and lanolin but without the addition of acid fast bacilli, complement-fixing antibodies against rabbit brain were first observed on the 20th day after injection, a slight decrease in titer was seen on the 27th, and no antibodies were demonstrable on the 41st day. Against human brain, antibodies were observed at about the same time as antibodies against rabbit brain, but the titer of the former was much lower. No antibodies fixing guinea pig brain were detected at any time after inoculation. In animals injected with two human-brain fractions, as described under item 3, no antibodies against nonfractionated human brain and guinea-pig brain antigens were detected on the 13th day after injection, but 2 weeks later almost all of the guinea-pig sera showed titers ranging from 1:4 to 1:32 against human-brain antigen and slightly lower titers against guinea-pig brain antigen.

From the foregoing it seems reasonable to consider the appearance of complement-fixing antibodies as a constant phenomenon in guinea pigs injected with brain substance incorporated into adjuvants. This is in contrast to some previous studies in which inoculation of adjuvant-brain emulsions into guinea pigs failed to elicit antibodies,¹² or their presence was demonstrable only in 1 of 3 animals.¹ These antibodies are apparently not species-specific. The addition of acid fast bacilli to the emulsion of brain tissue definitely enhances formation of antibodies and broadens

⁹ Jervis, G. A., Ferraro, A., Kopeloff, L. M., and Kopeloff, N., *Arch. Neurol. and Psychiatr.*, 1941, 45, 733.

¹⁰ Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, 74, 409.

¹¹ Kolmer, J. A., and Boerner, F., *Approved Laboratory Technic*, Chap. 29, 3rd edition, Appleton Century Co., New York, 1941.

¹² Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1947, 57, 229.

their immunological pattern, but apparently it is not an indispensable factor. The formation of antibrain antibodies was described by several groups of investigators¹³⁻¹⁶ who used as antigen brain tissue without adjuvants. However, inclusion of adjuvants in the inoculum seemed to facilitate the formation of antibodies.¹⁷ The lack of any significant relation between the clinical status of the animal and the level of antibrain precipitins was noted also by Kopeloff and Kopeloff¹² in rabbits.

(b) Antibrain antibodies were determined in animals first injected subcutaneously and then intracerebrally, as described under item 4. In almost all, the titers of antibrain antibodies obtained after the subcutaneous inoculation showed an increase following the intracerebral inoculation. However, there appeared to be no correlation between the clinical status of the animals and the level of antibodies before and after the intracerebral inoculation. Guinea pigs which had shown no antibrain antibodies after the subcutaneous inoculation with guinea pig brain, did develop antibodies following the intracerebral inoculation of the same antigen. A rise in antibody level after intracerebral injection was noted also in those animals which had been injected subcutaneously with rabbit brain suspension combined with adjuvants but without *Mycobacterium tuberculosis*. In the control experiments, no complement-fixing antibodies were detected in 10 nonsensitized guinea pigs inoculated intracerebrally with 0.1 ml of 10% rabbit-brain suspension.

These serological findings, although not directly related to the clinical status of the animal, may be correlated with the pathological data reported above, which indicate that the difference in the histopathological lesions observed in sensitized guinea pigs that died or survived after the intracerebral inocu-

lation was one of degree rather than quality. Possibly the increase in titer of antibrain antibodies is due to the release in the previously sensitized animal of small amounts of antigen from the brain tissue as a result of some microscopic injury suffered at the time of the intracerebral inoculation. However, this latter hypothesis requires further experimentation before any conclusions are warranted. The intracerebral "challenge" technic of previously sensitized guinea pigs, described above, may prove of value in the investigation of allergenic or paralitogenic properties of normal brain tissue or its fractions. This technic may be further broadened by injecting either non-fractionated homologous brain tissue, or purified fractions of the brain substance into animals previously sensitized by the subcutaneous route. Clinical observations of the animals, coupled with serological study, may throw additional light on the puzzling phenomenon of the allergenic properties of brain tissue.

In the discussions of the various aspects of the experiments reported above, reference to any possible application of the findings to human pathological conditions has been purposely avoided. Further experimental work is essential before one is justified to draw any conclusions applicable to conditions observed in human subjects, which could be attributed to the allergenic properties of brain tissue.

Summary. Allergic encephalitis in guinea pigs was produced by inoculation of brain tissue derived from 5 different mammalian species. Dilutions of phenolized rabies vaccines combined with adjuvants also caused paralysis of the inoculated animals. The majority of the guinea pigs previously sensitized by subcutaneous injection of brain tissue incorporated into adjuvants died after the intracerebral inoculation of homologous brain tissue (but not when brain tissue ofavian origin was used). Pathologic changes observed in the animals were described. Complement-fixing antibrain antibodies were found in sera of guinea pigs inoculated subcutaneously with brain tissue. Intracerebral reinoculation of these animals usually resulted in the rise of titer of the humoral antibrain antibodies.

¹³ Witebsky, E., and Steinfeld, J., *Z. f. Immunitätsf. u. Exp. Therap.*, 1928, **58**, 271.

¹⁴ Lewis, J. H., *J. Immunol.*, 1933, **24**, 193.

¹⁵ Schwentker, F. F., and Rivers, T. M., *J. Exp. Med.*, 1934, **60**, 559.

¹⁶ Bailey, G. H., and Gardner, R. E., *J. Exp. Med.*, 1940, **72**, 499.

¹⁷ Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1944, **48**, 297.

Bactericidal Action of Streptomycin-Penicillin Mixtures *In vitro*.

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Studies were carried out in order to determine whether streptomycin and penicillin in combination were synergistic in their bactericidal action against 4 common organisms. Synergism is defined for the purpose of this paper as a cooperative action greater than would be predicted were the action of the agents mathematically additive.

Methods. On the basis of preliminary experiments, all media were adjusted to a sodium chloride concentration of 0.25% and to a pH of 7.6. All tests were performed in beef infusion broth (Difco) containing 1% neopeptone. The bacteria were grown at 37°C and a 6-hour culture was diluted so as to contain approximately 500,000 bacteria per/ml of media. One ml of this dilution was used as the inoculum.

Test organisms were *Staphylococcus aureus* Oxford H, *Staphylococcus aureus* No. 209, *Staphylococcus aureus* SM, and *Streptococcus hemolyticus* C-203 MV. All organisms were tested separately for susceptibility to penicillin and streptomycin. Titration of penicillin was made in 2 ml of medium and 1 ml of standard inoculum was added. The cultures were inoculated at 37°C. Readings were made at 24 hours compared with a bacterial control and subcultures were made of each tube to blood agar to determine the presence of growth. Streptomycin titrations were made in the same manner. This method determined the minimum lethal concentration of penicillin and of streptomycin for each organism. In each instance it was also determined that growth did occur in concentrations of 75% or more of this minimum lethal concentration.

Using the minimum lethal concentration as a guide, titrations of the combined antibiotics were set up. A penicillin titration was made to which varying amounts of streptomycin were added. Penicillin, streptomycin, and

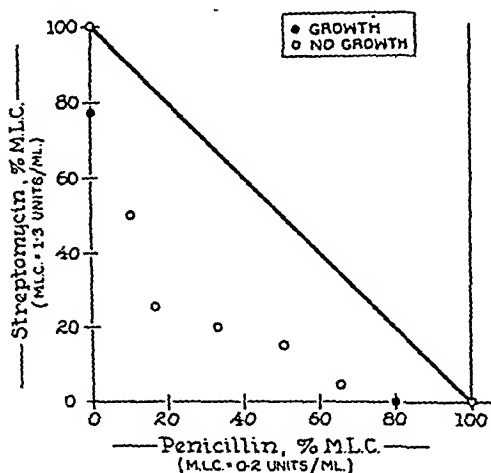


FIG. 1.
Staphylococcus aureus Oxford H.

bacterial controls were set up with each titration. Subcultures were made to blood agar and compared with subcultures of the antibiotic control.

Experimental Results. Fig. 1 is a typical graph for *Staphylococcus aureus* Oxford H. The minimum lethal concentration of penicillin was 0.2 unit per ml and for streptomycin was 1.3 units per ml. The diagonal line represents bactericidal combinations of penicillin and streptomycin which should be bactericidal if these two antibiotics were additive in their effect. For any given concentration of penicillin, the value representing the smallest amount of streptomycin which rendered the mixture bactericidal is plotted on the graph as a circle. For instance in this case, when the dose of penicillin was reduced to 16% M.L.C., the mixture was bactericidal when 25% M.L.C. of streptomycin was present.

In Table I are presented the results of similar studies on three strains of staphylococci and one strain of streptococci.

TABLE I.
Bactericidal Combinations of Penicillin and Streptomycin for Various Pure Cultures.

Organism	% M.L.C.* Streptomycin	% M.L.C.* Penicillin	Sum
Hemolytic streptococcus C-203 MV	5.6	50.7	56.3
	10.0	25.3	35.3
	14.3	23.0	37.3
	28.7	15.3	44.0
	43.4	5.0	48.4
	56.5	2.5	59.0
<i>Staphylococcus aureus</i> No. 209	11.0	76.0	87.0
	13.0	61.0	74.0
	14.0	50.0	64.0
	28.0	38.0	66.0
	43.0	25.0	68.0
	56.0	12.0	68.0
<i>Staphylococcus aureus</i> Oxford H	15.3	50.0	65.3
	20.0	33.0	53.0
	25.3	16.0	41.3
	50.7	10.0	60.7
<i>Staphylococcus aureus</i>	10.0	76.1	86.1
	15.3	50.7	66.0
	25.3	20.0	45.3
	76.1	10.0	86.1
<i>Staphylococcus aureus</i> SM	10.0	76.1	86.1
	15.3	25.3	40.6
	25.3	20.0	45.0
	50.7	15.3	66.0
<i>Staphylococcus aureus</i> SM	10.0	50.7	60.7
	15.3	25.3	40.6
	25.3	20.0	45.3
	50.7	15.3	66.0
	76.1	10.0	86.1

* M.L.C. Minimum Lethal Concentration of each antibiotic alone for the particular organism determined to within 25% (i.e., the organisms are known to grow at 75% M.L.C. or higher.)

Summary. Under the experimental conditions used, definite synergism was noted in the bactericidal action of penicillin and streptomycin upon staphylococci and streptococci *in vitro*.

16761 P

A Complement Fixation Test for Dengue.*

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Studies carried out by one of us (A. B. S.) since 1944 have established the fact that several immunologically distinct but related types of virus are responsible for the clinically typical and atypical forms of dengue.¹ Since

the adaptation of dengue virus to mice,² Sabin and Schlesinger found that the neutralizing antibodies which developed following infection were so highly type-specific, that by means of the neutralization test³ in mice one

could not diagnose infections caused by heterologous immunological types of virus which have not as yet been propagated successfully in mice. Continuous serial, intracerebral passage in mice has increased the titer of our Hawaii strain of virus from 10^{-1} to about 10^{-5} or 10^{-6} , and following intracerebral passage in 3- to 4-day-old mice (a procedure suggested by Dr. Gordon Meiklejohn) titers of 10^{-7} to 10^{-8} could be achieved. Satisfactory complement-fixing (C-F) antigens could be prepared from the brains of mice which were either 3 to 7 days old or approximately 14 days old at the time of inoculation with virus of highest titer (0.03×10^{-7} – 0.03×10^{-8}) which could be obtained only from the younger suckling mice. Comparative tests on aliquot portions of 20% aqueous extracts of the infected mouse brains revealed that antigen prepared by benzene extraction of the lyophilized material^{4,5} was no more potent as regards the number of antigenic units or the titer obtained with dengue antisera than that prepared only by centrifugation at 13,000 r.p.m. in the cold (Type SS-1 Swedish angle centrifuge—Sorvall), and both were equally effective in increasing the titer of complement. The 20% mouse brain suspension prepared by inoculation of 3- to 7-day-old mice contained 16 to 32 units of antigen per 0.25 ml as compared with 4 units (with two exceptions when it was only 1 unit and one when it was 8 units) for the similar suspension prepared in a similar manner from approximately 14-day-old

mice. Almost all the work to be reported was carried out with benzene extracted antigens (containing 4 units), which, however, also had to be centrifuged at 13,000 r.p.m. for 1 hour to remove all the anticomplementary properties. The tests were set up in the usual manner, using 2 exact units of complement, and incubation for 16 to 20 hours in a refrigerator at about 5°C. The titers represent the highest original dilution of a serum, *i.e.*, before addition to the mixture, which yielded at least 2-plus fixation. Similarly prepared extracts from normal mouse brains were used as controls in all the tests.

Results. (1) Human volunteers, rhesus monkeys, and chimpanzees⁶ inoculated with the homologous Hawaii strain of the human virus, or with immunologically identical human strains, developed C-F antibodies which reached peak titers of 1:64 to 1:256 within 2 to 6 weeks. These antibodies have been found to persist in high titer for many months, and in human volunteers, residing in dengue-free regions of the U. S. A., titers of 1:4 to 1:128 have been found 38 months after infection, and titers of 1:4 to 1:16 as long as 52 months' after infection, the longest period tested.

(2) Human volunteers inoculated with immunologically distinct strains of human dengue virus, who developed little or no neutralizing antibodies for the Hawaii virus,¹ nevertheless, developed C-F antibodies in titers no higher than 1:16; however, 6 months after inoculation these antibodies were not demonstrable in serum dilutions of 1:4 in some of the individuals tested.

(3) Rhesus monkeys inoculated with immunologically distinct strains of human dengue virus, which developed little or no neutralizing antibodies for the Hawaii virus, nevertheless developed C-F antibodies in titers as high as 1:512; however, the fixation was not complete (*i.e.*, only 2- to 3-plus) even in the lowest dilutions of such sera, suggesting that only about 1 unit of the serologically related component was present in the antigens used. Furthermore, in some of the monkeys tested,

* This investigation was aided by the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of The Surgeon General, Department of The Army.

† Lt., Medical Corps, A.U.S., on active duty.

¹ Sabin, A. B., in *Viral and Rickettsial Infections of Man*, edited by T. M. Rivers, J. B. Lipincott Co., Philadelphia, 1948.

² Sabin, A. B., and Schlesinger, R. W., *Science*, 1945, 101, 640.

³ Sabin, A. B., *Diagnostic Procedures for Virus and Rickettsial Diseases*, Am. Pub. Health Assn., 1948, 289-293.

⁴ DeBoer, C. J., and Cox, H. R., *J. Immunol.*, 1947, 55, 193.

⁵ Espana, C., and Hammon, W. McD., *J. Immunol.*, 1948, 59, 31.

⁶ Paul, J. R., Melnick, J. L., and Sabin, A. B., *Proc. Soc. Exp. Biol. and Med.*, 1948, 68, 193.

thus far, these antibodies disappeared as early as 6 to 10 weeks after inoculation.

(4) Rhesus monkeys inoculated intracerebrally with the homologous *mouse-adapted* Hawaii virus, developed C-F antibodies in titers as low as 1:16 which in some monkeys disappeared as early as 6 weeks after inoculation, although neutralizing antibodies in high titer persisted for many months.

(5) Human volunteers inoculated with dengue vaccine, consisting of the living mouse-adapted Hawaii virus, who developed neutralizing antibodies and resisted infection with the unmodified, human virus^{1,7} failed to develop C-F antibodies.

(6) Sera obtained from individuals 2 years after a natural attack of dengue in Hawaii or Japan, which neutralized the Hawaii virus in high titer, also fixed complement in titers of 1:16 to 1:64. Positive results also have been obtained with sera of individuals who had dengue in Singapore, Java, and New Guinea.

(7) Sera collected from American marines,

⁷ Sabin, A. B., and Schlesinger, R. W., unpublished studies.

11 months after a single, primary natural attack of dengue on Guam, gave positive C-F tests in titers of 1:2 to 1:8 with the Hawaii antigen, although they had no significant neutralizing antibodies⁷ for this virus.[†]

(8) Human volunteers infected with phlebotomus (pappataci, sandfly) fever virus developed no C-F antibodies for the dengue antigen.

(9) Rhesus monkeys which had no C-F antibodies for dengue 7 weeks after inoculation with the French neurotropic yellow fever virus, developed such antibodies in titers of 1:4 to 1:32, 8 to 10 days after a large booster dose of either the Asibi viscerotropic or French neurotropic strains of yellow fever virus.[§] These results indicate an antigenic, if not an immunogenic,¹ relationship between the viruses of yellow fever and dengue.

[†] We are indebted to Drs. R. E. Shope and Horace Hodes, at the time serving with the U. S. Naval Medical Research Unit No. 2, for obtaining, lyophilizing and forwarding these sera in 1945.

[§] We are indebted to Dr. Max Theiler, of the International Health Division of the Rockefeller Foundation, for the sera from these monkeys.

16762

Influence of Choline, Cystine and Methionine on Toxic Effects of Pyridine and Certain Related Compounds.

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(With the technical assistance of Lucy C. Gremillion.)

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In a study of the pharmacological effects produced by the ingestion of various pyridine derivatives by the rat^{1,2} pyridine and coramine (nikethamide) were found to be the most active from the standpoint of the production of marked changes in liver size and composition. Coramine produced a great increase in liver weight without causing very much damage to the liver or modifying its composition. Pyridine has proved to be somewhat more toxic than many of its derivatives^{1,3} and it must be included in the list of sub-

stances known to produce severe liver and kidney damage.^{4,5} Methionine and cystine have been shown to have some liver protective

¹ Coulson, R. A., and Brazda, F. G., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 1.

² Brazda, F. G., and Coulson, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 37.

³ Brazda, F. G., and Coulson, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 19.

⁴ Baxter, J. H., *Proc. Am. Fed. Clin. Research*, 1945, **2**, 77.

⁵ Baxter, J. H., *Am. J. Path.*, 1948, **24**, 503.

action when included in the diet of rats receiving pyridine.^{4,5}

This report deals with the quantitative effects of the daily ingestion of pyridine or several heterocyclic compounds belonging to the quinoline series. The influence of various liver protective agents is also reported.

Experimental. The experimental animals consisted of equal numbers of male and female rats from our stock colony. The rats were placed on the test or control diets a few days after weaning. The basal diet had the same composition as that used for the coramine studies.² This diet consisted of 25% casein, 55% starch, 15% cottonseed oil and 5% salts. Five hundred ml of boiling water were added to each kg of dry diet. Each rat was given one ml of brewer's yeast extract a day and 3 drops of Percomorph oil a week.

The livers and kidneys were removed, weighed, and a small section was saved for histological examination. The livers were dried *in vacuo* and fat and water contents were determined as described by Brazda and Coulson.² Every effort was made to obtain the livers and kidneys for analysis from rats that had become moribund and had succumbed to the effect of the more toxic diets. Results obtained from these animals were so nearly alike regardless of the length of time the animals were on the diet that the data of all the moribund rats in a given experiment were gathered together into one group. In order to eliminate the effects of postmortem changes in the composition of the liver the tables presented do not include the results from any animal that had been dead for over 10 minutes. Records were kept of the growth rate, the food consumption, and length of life of the experimental animals.

In all, about 1200 rats were placed on diets containing pyridine or quinoline (in the form of acetates) to which various supplements suspected of being liver protective were added. Each rat received a fresh lump of the experimental diet each day and any diet left over from the previous day was discarded. The animals were weighed at the beginning of the

experiment and at the time of death or at the time they were killed for autopsy. In all cases the experiments were concluded at 28 days. The experiments were conducted at an average temperature of about 75°F. As the season progressed the animals were removed to a room where the temperature was maintained at 76°F and the relative humidity at 50%.

Pyridine Series. Each group contained 70 rats, one female and one male from each of 35 litters. As far as was possible each litter contributed rats to 3 experimental groups. Group I received a diet which contained 0.6% pyridine; group II 0.6% pyridine plus 0.5% choline; group III 0.6% pyridine plus 1% cystine; group IV 0.6% pyridine plus 1.2% methionine; group V 0.6% pyridine plus 0.6% beta picoline; and group VI 0.6% pyridine plus 1.0% nicotinamide. In spite of the unpleasant odor of the diet the rats ate it willingly. There was no great difference in either food consumption or growth rate in the first 4 groups. The addition of beta-picoline or nicotinamide greatly reduced food intake which in turn reduced the growth rate. Two other pyridine groups were set up to determine the reproducibility of the survival curves. These groups are not included in Table I since there was no significant variation in the shape or the absolute area contained within the curve and hence it was concluded that 70 rats were sufficient for testing the effect of pyridine on survival.

The results are summarized in Fig. 1. Probably the best index of the relative survival time is obtained by comparing the respective areas under the curves. Considering a control curve to have an area of 1000, then the pyridine curve has an area of 348, the pyridine plus choline 465, the pyridine plus cystine 706, and pyridine plus methionine 773. It is apparent that methionine and cystine are far more effective in prolonging the life of the experimental animals than choline in spite of the fact that choline is known to be a lipotropic compound whereas cystine is ineffective in this respect. These results are in agreement with those reported by Baxter.⁶ The survival curves of the groups receiving supplements of beta-picoline or nicotinamide in addition to the pyridine are not included in

⁶ Baxter, J. H., *J. Clin. Invest.*, (Proc.). 1946, 25, 908.

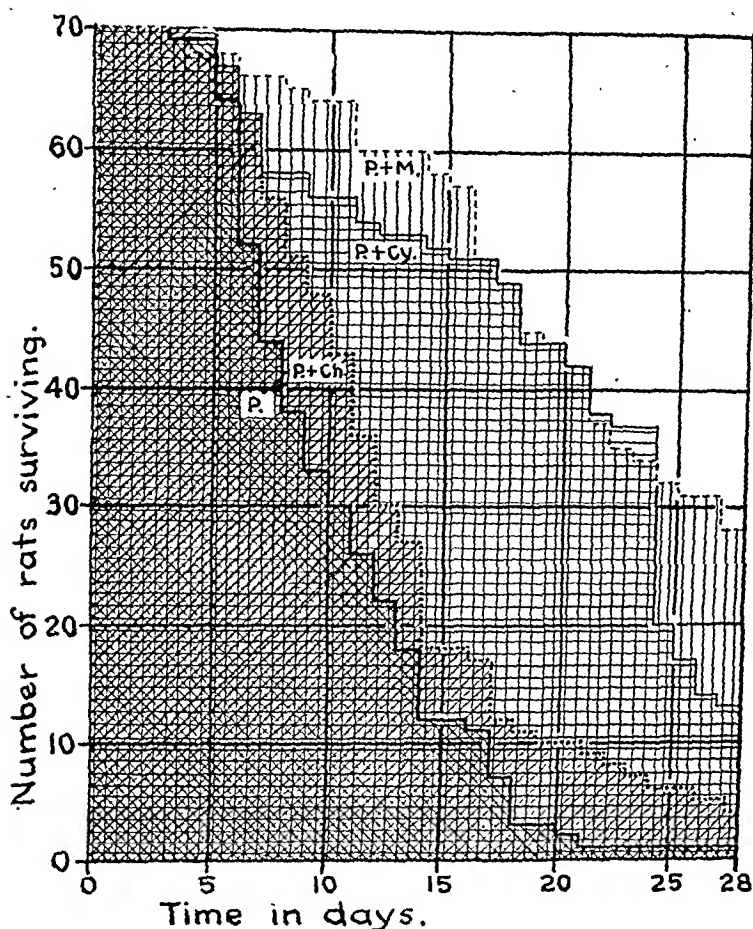


FIG. 1.

Survival of Rats on Pyridine Diets. P = 0.6% pyridine; P + Ch = 0.6% pyridine plus 0.5% choline; P + Cy = 0.6% pyridine plus 1.0% cystine; P + M = 0.6% pyridine plus 1.2% methionine.

Fig. 1. Both groups of animals ate much less and grew at a correspondingly lower rate which introduced the factor of food consumption. The area under the curves was greater than that found in the pyridine or pyridine and choline groups but much less than that found in the pyridine and cystine or pyridine and methionine groups. Paired feeding experiments are necessary to evaluate the influence of these two compounds since a decreased food intake means a correspondingly decreased intake of pyridine.

Table I presents the results of the liver analyses for the 6 groups of 70 rats each and for 10 rats which were fed piperidine, the reduced derivative of pyridine. The table

may be summarized by stating that all of the rats in the pyridine experiments had marked liver enlargement. Without exception the moribund rats had livers which were larger than those of the sacrificed animals and which had a greatly decreased solid content. There are no significant differences in the growth rate of the animals receiving pyridine alone of those receiving additional supplements of choline or methionine. The cystine series grew at a rate somewhat below that of the others. Although the food intake and the growth rate paralleled each other rather closely both were subject to considerable individual variation.

The average fat contents of the pyridine

TABLE I.
Effect of Various Compounds on Size and Composition of Livers of Rats Receiving Pyridine.

Group	No. rats	No. days	Liver wt as % body wt		Liver fat, % dry wt	Liver % solids, avg	Gain in wt, g/day
			Avg	P.E.*			
0.6% pyridine—Sacrificed	11	5	6.21	±0.25	17.36	31.26	+1.42
	10	9	6.39	±0.12	21.33	33.05	+2.33
0.6% pyridine—Moribund	12	—	8.96	±0.24	24.14	25.08	—
0.6% pyridine + 0.5% choline—Sacrificed	10	5	5.84	±0.18	13.24	29.38	+1.81
	13	9	5.79	±0.12	11.49	28.23	+1.99
	2	28	5.57	—	11.76	31.03	+2.15
0.6% pyridine + 0.5% choline—Moribund	18	—	8.41	±0.17	12.00	22.91	—
0.6% pyridine + 1% cystine—Sacrificed	12	28	9.75	±0.33	16.23	26.52	+1.31
0.6% pyridine + 1% cystine—Moribund	5	—	10.33	±0.31	13.03	24.53	—
0.6% pyridine + 1.2% methionine—Sacrificed	11	5	5.89	±0.11	14.89	31.29	+1.13
	12	9	7.03	±0.16	16.17	28.75	+1.86
	28	28	6.50	±0.11	10.38	29.00	+1.97
	10	—	9.50	±0.13	12.14	22.91	—
0.6% pyridine + 1.2% methionine—Moribund	9	28	8.77	±0.25	17.19	31.11	+1.18
0.6% pyridine + 0.6% beta picoline—Sacrificed	7	—	9.44	±0.37	21.02	24.69	—
0.6% pyridine + 0.6% beta picoline—Moribund	17	28	7.96	±0.31	14.78	28.70	+0.57
0.6% pyridine + 1.0% nicotinamide—Sacrificed	7	—	10.44	±0.24	16.47	25.38	—
0.6% pyridine + 1.0% nicotinamide—Moribund	10	28	4.93	±0.14	7.59	28.46	+1.30
0.6% piperidine	10	5	4.98	±0.09	13.27	29.21	+3.21
	10	9	4.78	±0.09	13.38	29.15	+3.30
Control	10	28	5.18	±0.13	10.89	29.48	+3.79

$$* \text{P.E.} = 0.6745 \sqrt{\frac{\sum(v)^2}{n(n-1)}}$$

TABLE II.
Effect of Injection of a Lethal Dose of Pyridine on the Water Content of the Rat Liver.

Group	No. rats	Final wt avg	Liver wt as % body wt		Liver fat, % dry wt	Liver % solids, avg
			Avg	P.E.		
Experimental	18	49.5	4.90	± 0.11	8.66	25.40
Controls	18	57.6	4.78	± 0.17	14.62	32.60

and pyridine plus cystine groups are slightly higher than those of the groups receiving the lipotropic factors choline or methionine. The high protein diet is instrumental in preventing an excessive accumulation of fat in the livers of all groups. Piperidine had no noticeable effect on the liver although it did decrease food intake and growth rate.

Nine males and 9 female rats whose average age was 25 days were injected subcutaneously with pyridine in amounts that were calculated to be 3 times the LD_{50} .³ These animals died in from 1 to 4 hours and the livers were removed, analyzed, and a piece was saved for histological study. The results appear in Table II. No significant liver enlargement was noted although the livers did show an increase in the average water content. The solid content was still considerably above the solid content found for the moribund group in Table I. In general the picture presented by the group injected with lethal doses of pyridine is quite different from that of the rats which were fed pyridine. It is probable these animals died from respiratory paralysis rather than from liver failure.

Quinoline Series. The survival curves found in Fig. 2 were obtained in the same manner as those of the pyridine experiments. Quinoline was used at a dietary level of 1% which is equivalent to 0.6% pyridine. If the control curve is given an arbitrary value of 1000 the relative area under the methionine curve is 812, the cystine is 745, the choline is 648, and the quinoline alone is 535. The fact that the relative order of effectiveness of the liver protective compounds is the same as in the pyridine groups suggests that the same mechanism is involved in both experiments. Methionine is again somewhat superior to cystine. Since the addition of choline does prolong the life of the quinoline rats it is probable that the slight difference between

the pyridine and pyridine and choline groups in Fig. 1 is significant.

The results of the liver analyses are presented in Table III. The livers are very greatly enlarged in all 4 groups, approaching one-seventh of the total body weight. Although none of the rats presented in this table were moribund, the values for the liver solid content of the quinoline and the quinoline and choline groups closely resemble the figures obtained from the moribund rats of the pyridine groups. Without resort to histological examination it is apparent that cystine and methionine retard or prevent the hydration of the livers.

In the pyridine series it was observed that neither methionine nor cystine had any beneficial effect on the rate of growth of the animals on the diet reported in this paper. Quinoline alone retards the growth rate seriously and the addition of choline does not prevent this retardation. However the inclusion of either cystine or methionine increases the growth rate of the animals in a dramatic fashion. In the entire series of rats receiving quinoline with or without choline not one rat gained weight over the 28-day period. Of the 99 rats receiving quinoline plus cystine or methionine which survived long enough to be autopsied only 2 failed to gain weight. The growth rate was commensurate with the food intake which means that although those rats which received cystine or methionine ingested more quinoline, they remained in better health and lived longer than those of the other two groups.

A number of animals that had become moribund after eating diets which contained quinoline were examined immediately after death. It was not possible to obtain enough of these for the results to be of statistical significance. Those animals that died in the first few days had eaten so little of the diet that starvation

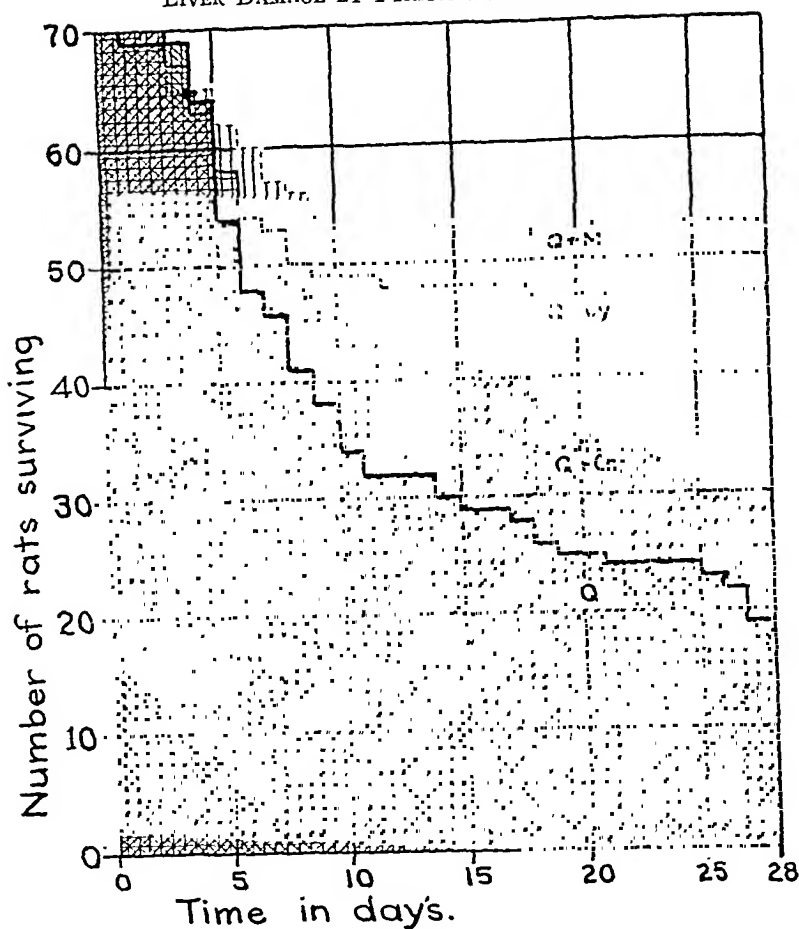


FIG. 2.

Survival of Rats on Quinoline Diets. Q = 1.0% quinoline; Q + Ch = 1.0% quinoline plus 0.5% choline; Q + Cy = 1.0% quinoline plus 1.0% cystine; Q + M = 1.0% quinoline plus 1.2% methionine.

probably contributed to a large extent to their death. In spite of this, gravimetric analyses of the livers of these few moribund rats gave results which were similar to those from the moribund animals of the pyridine groups. The very great increase in the liver weight of the sacrificed rats in the quinoline and quinoline and choline groups reported in Table III indicates that all of these experimental animals ate enough of the diet to produce liver damage.

On the basis of the theory that the tertiary nitrogen of quinoline is responsible for its action on the liver, quinoline was methylated to quinoline methochloride and this derivative was fed to 5 male and 5 female rats. The

methochloride proved to be extremely toxic at a dietary level of 1.39% which is equivalent to a 1% quinoline level. Seven of the 10 rats died in less than a week; the other 3 were arbitrarily killed at 7 days and examined. The livers were normal in every respect and the experiments with this compound were not continued.

Isoquinoline at a level of 1% of the diet was also very toxic. Of the 20 rats that were fed this substance only 7 remained alive at 5 days. These 7 had livers which were hydrated to some extent although the degree of the liver enlargement was not marked. Isoquinoline methochloride was as toxic as isoquinoline but it produced no noticeable effect on liver size.

TABLE III.
Effect of Choline, Cystine, or Methionine on Livers of Rats Receiving Quinoline.

Group	No. rats	No. days	Liver wt as % body wt		Liver fat, % dry wt	Liver % solids, avg	Gain in wt, g/day
			Avg	P.E.			
1.0% quinoline	18	28	11.27	±0.24	11.62	24.10	-0.40
1.0% quinoline + 0.5% choline	28	28	14.69	±0.10	18.21	25.92	-0.63
1.0% quinoline + 1.0% cystine	47	28	14.49	±0.13	14.49	29.69	+0.39
1.0% quinoline + 1.2% methionine	52	28	11.09	±0.16	11.15	28.55	+0.36

or composition.

Histological Appearance of the Livers. The ingestion of pyridine causes the production of cirrhosis or more commonly necrosis or both in the young rat. This is in agreement with results recently reported by Baxter.⁵ On histological examination the livers of the moribund rats appeared to be hydrated and showed many small irregular vacuoles which were similar to those described by Trowell.⁷ Gravimetric analyses of the whole livers, which usually reveal more information than does microscopic examination of small sections, clearly showed not only the presence of extra water but also the magnitude of its increase. In animals that died during the experiments with pyridine the histological picture of the livers was the same regardless of the presence or absence of a liver-protective supplement. The apparently healthy rats that were killed arbitrarily at different time intervals had livers which showed little damage. It would appear, therefore, that the development of severe necrosis following the ingestion of pyridine requires a relatively short period of time. Damage to the kidney is roughly but not absolutely proportional to the damage to the liver. Although many cases of kidney damage were seen few of these were severe enough to be considered as the primary cause of death and it was concluded that most of those animals that died from the ingestion of pyridine died from liver failure. Further evidence that liver failure was the cause of death may be obtained from consideration of the fact that all of the moribund rats had livers which were very severely damaged as shown by gravimetric analysis. The injection of lethal

doses of pyridine into young rats had no effect on the histological appearance of the livers.

The animals of the quinoline series had livers which were more nearly normal than those in the pyridine groups as far as the microscopic picture was concerned. Although the cells were swollen and some small areas of necrosis were seen the overall appearance of the liver was not suggestive of marked damage. The percentage liver weights were from 2 to 2½ times the control weights, yet the extent and nature of the enlargement was not apparent from the slides. It is by no means certain that rats which died while receiving quinoline in the diet died of liver failure.

Discussion. The survival curves are useful in determining the toxic effects of compounds of the pyridine or quinoline nucleus and the influence of certain agents that reduce the toxicity of these substances. The sulfur containing aminoacids, methionine and cystine, are superior to choline in preventing liver damage. The fact that cystine is a good protective agent suggests that transmethylation is probably not involved in this protection since there is no evidence that cystine can contribute anything to methylation of the ring nitrogen as a means of detoxication. Although pyridine and quinoline may be methylated by the rat there is still no conclusive evidence that this is the case.¹ It is possible that the sulfur-containing compounds are involved in the detoxication of quinoline and pyridine in a manner as yet unknown.

Trowell⁷ has reported that intact rats whose livers have been subjected to procedures designed to produce anoxia without reduction

⁷ Trowell, O. A., *J. Physiol.*, 1946, 105, 268.

of the intrasinusoidal pressure develop watery vacuoles in this organ. Inasmuch as the liver in a rat dying from effects of pyridine becomes hydrated, especially during the last hours of life, it seems logical to assume that this is due basically to a decrease in the rate of oxidation in the cells.

Summary. Pyridine alone or pyridine supplemented with either choline, cystine, methionine, nicotinamide or beta-picoline was fed to young rats on a high protein diet. Livers from rats dying from the effects of pyridine are enlarged and always exhibit a marked

increase in water content. Livers from apparently healthy animals which have been on a pyridine diet show a nearly normal water content and are enlarged. Cystine and methionine afford protection against the effects of pyridine. The feeding of quinoline alone or quinoline supplemented with either choline, cystine, or methionine produced enlarged livers. Cystine and methionine increased survival time. Choline showed some protective effect. Cystine or methionine prevented the accumulation of water in the livers of rats fed quinoline. Choline had no effect.

16763

Quantitative Detection of Minute Concentrations of Digitoxin.*

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The detection of a digitalis glycoside, Lanatoside C, in various media by means of the embryonic duck heart preparation has been reported in previous studies.¹⁻³ It was found in these same studies that not only could extremely minute amounts of the glycoside (less than 0.1 μg per cc) be detected by this preparation, but also quantitative estimations of the actual amount of glycoside in Tyrode's solution or serum could be determined.

However, the sensitivity of the embryonic duck heart to the digitalis glucoside, digitoxin, had not been determined. Accordingly, studies were made of the effect of various concentrations of digitoxin upon the duck heart immersed in (1) Tyrode's solution, (2) rat serum, and (3) human serum. The method of quantitation employed was the

same as that described previously.^{1,3} It was found that the embryonic duck heart was not only extraordinarily sensitive to minute quantities of digitoxin but also offered a means whereby the concentration of digitoxin could be assessed in a quantitative fashion.

Results. As Table I demonstrates, the embryonic heart preparation was able to detect the presence of 0.005 μg of digitoxin in one cc of Tyrode's solution. Moreover, with increasing concentrations of the drug, there was a progressive reduction in the time taken for the occurrence of the digitalis effect.

The action of digitoxin in rat serum was found to be much less effective than in Tyrode's solution. Thus (Table I) only quantities of 0.2 μg or more of digitoxin per cc could be detected. Human serum was even more inhibitory (Table I) in that only quantities of 0.60 μg or more of digitoxin per cc could be detected. This action of serum upon the action of digitoxin also has been demonstrated by previous observers.^{4,5} Lanatoside C, how-

* Aided by grants from the Life Insurance Medical Research Fund, The Public Health Service and The Sandoz Chemical Works.

¹ Friedman, M., and Bine, R., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 162.

² Friedman, M., and Bine, R., Jr., *Am. J. Med. Sci.*, 1947, **214**, 633.

³ Bine, R., Jr., and Friedman, M., *Am. J. Med. Sci.*, 1948, **216**, 534.

⁴ Fawaz, G., and Farah, A., *J. Pharm. and Exp. Therap.*, 1944, **80**, 193.

⁵ Suter, E., *Helvet. Physiol. et Pharmacol. Acta*, 1944, **2**, 2.

TABLE I.

The Detection of Digitoxin in (1) Tyrode's Solution, (2) Rat Serum, and (3) Human Serum by the Embryonic Duck Heart Preparation.

Conc. of digitoxin ($\mu\text{g/cc}$)	Tyrode's solution			Rat serum			Human serum		
	Avg. time "dig. effect" (min.)	Stand. error of mean (min.)	No. hts.	Avg. time "dig. effect" (min.)	Stand. error of mean (min.)	No. hts.	Avg. time "dig. effect" (min.)	Stand. error of mean (min.)	No. hts.
.001	ND		10	ND		12	ND		10
.005	48	3.0	15	ND		10	ND		10
.010	41	1.6	21	ND		10	ND		10
.05	23	1.5	33	ND		10	ND		10
.10	12	0.8	20	ND		10	ND		10
.20	—	—	—	62	1.3	18	ND		15
.40	—	—	—	38	1.7	18	ND		15
.60	6	0.2	20	24	0.5	36	49	2.0	23
.80	—	—	—	21	0.8	20	34	1.6	24
1.00	4	0.2	29	11	0.4	22	21	1.1	28

ND indicates that no glycoside could be detected by occurrence of "digitalis effect" in embryonic heart preparation.

ever, was not found³ to be inhibited similarly by serum.

Conclusions. 1. Quantitative assay of extremely minute amounts of digitoxin in

Tyrode's solution and in rat and human serum was found to be possible by means of the embryonic duck heart preparation.

16764

Peculiar Enlargement of Eyeballs in Chicks Caused by Feeding a High Level of Glycine.*

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Previous reports^{1,2} from this laboratory have shown that glycine is highly "pellagra-genic" when fed to chicks receiving a nicotinic acid-low diet. On the other hand, the chick tolerated unusually high levels of glycine (4 to 6%), provided a sufficient amount of nicotinic acid was contained in the diet.²

The latter observation was somewhat surprising for it had been reported earlier that more than 2% of glycine was toxic to the young chicken³ and that 4 g of glycine per day were harmful to hens.⁴

Because of this discrepancy, an investigation was undertaken to determine the minimum toxic level of glycine and the extent to which this level could be influenced by nicotinic acid feeding. The present report describes a peculiar overgrowth of the eyeballs and other conditions observed in chicks resulting from feeding a highly purified diet con-

* Scientific Paper No. A202. Contribution No. 2119 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry).

† Now at Michigan State College, East Lansing, Mich.

‡ Now at University of Minnesota, St. Paul, Minn.

¹ Briggs, G. M., Groschke, A. C., and Lillie, R. J., *J. Nutrition*, 1946, **32**, 659.

² Groschke, A. C., and Briggs, G. M., *J. Biol. Chem.*, 1946, **165**, 739.

³ Almquist, H. J., Stokstad, E. L. R., Meechi, E., and Manning, P. D. V., *J. Biol. Chem.*, 1940, **134**, 213.

⁴ Patton, A. R., *Poultry Sci.*, 1939, **18**, 31.

TABLE I.
Composition of Basal Diet 113GN.

	%
Cerelose	71.4
Casein (crude)	18.0
Mineral mixture 1M	6.0
Soybean oil	4.0
dl-Methionine	0.3
Choline chloride	0.2
i-Inositol	0.1
Vitamins (mg/100 g).	
Thiamine HCl	0.4
Riboflavin	0.8
Ca pantothenate	2.0
Pyridoxine HCl	0.6
Biotin	0.02
Pteroylglutamic acid	0.2
Para-aminobenzoic acid	0.2
2-Methyl-1,4-naphthoquinone	0.1
Alpha-tocopherol	0.5
Vit. A and D ₃ , 1200 U.S.P. and 170 A.O.A.C. units, respectively, by dropper weekly.	

taining 8% of glycine.

Experimental. Day-old New Hampshire chicks of mixed sexes were used. The chicks were distributed according to weight, confined in electrically heated wooden cages with wire screen floors and given feed and water *ad libitum*. Weighings and other observations were made weekly. All substitutions in the basal diet 113GN were made at the expense of cerelose (Table I).

Results. Table II summarizes the results of feeding 8% of glycine to chicks in combination with various levels of nicotinic acid. It is quite evident that glycine at this level inhibits growth and that the growth depression is not overcome by feeding nicotinic acid in amounts as high as 100 mg per 100 g of diet. A few cases of perosis occurred in the groups receiving glycine and feathering was inferior. No "chick black-tongue" was seen, as might be expected with the high level of glycine fed.

During the first week of the experiment the chicks in the 3 groups receiving glycine exhibited periods of extreme prostration accompanied by tremors. This condition seemed to be especially severe after eating. Sometimes the chicks would become so comatose they would appear to be at the point of death at which time they would gradually rally. In spite of these symptoms, constant weight gains were made.

After the first week the chicks appeared to outgrow the tendency toward prostration, but slight tremors existed in the birds throughout the whole experiment. They seemed to be constantly fatigued and would stretch their wings and legs at frequent intervals.

At about the third week a peculiar puffiness about the eyes was noticed. By the fourth week it was evident that either the eyeballs were becoming enlarged or that an edemic condition existed in the optic vesicle which was causing the eye to be forced out, as occurs in exophthalmic goiter in the guinea pig. The eye condition at this point was severe enough to interfere with the normal movements of the nictitating membrane.

At the end of 4 weeks, 3 typical chicks from each group receiving glycine were selected and the total of 9 chicks were given the diet containing 8% of glycine and 10 mg of nicotinic acid per 100 g of diet. They were continued on experiment to 7 weeks of age along with the 6 control chicks. From the fourth week to the seventh week, the "popped" eye condition became worse. From about the fifth week on, the eyes were so bulged that normal vision was impaired to the point where it was evident that the eyes could not be focused on objects close at hand. Consequently, the feed hoppers had to be kept quite full so that the chicks could eat properly.

At 7 weeks of age all the chicks were killed by severing the jugular vein. All had been fasted for a period of 6 hours prior to killing. The heart, liver with bile sac, spleen, thyroids, and one eye from each chick were removed and weighed immediately. All adhering muscular tissue was cut away from the eyes before they were weighed. In Table III the average values obtained for the various organs are given expressed in terms of percent of body weight. The data show that on this basis the values of the 2 groups for the thyroids, heart, liver plus bile sac, and spleen were not appreciably different. However, a marked difference existed in the weight of the eyeballs. The eyes from the chicks which received 8% of glycine were almost twice as great on a percent-of-body-weight

TABLE II.
Effect of 8% of Glycine on Chicks Receiving Various Levels of Nicotinic Acid.

Group No.	Supplement of basal diet 113GN containing 5% gelatin	No. chicks	No. dead	Avg wt 4 wk, g	No. perosis	Feed efficiency*
1	10 mg % nicotinic acid	6	0	235	0	.510
2	10 " " " " " + 8% glycine	6	1	157	2	.501
3	50 " " " " " + " "	6	1	152	1	.551
4	100 " " " " " + " "	6	1	158	1	.504

* Total weight gained

= feed efficiency.

Total feed consumed

TABLE III.
Effect of 8% of Glycine in the Diet of Chicks on the Size of Various Organs of the Body.

Diet	No. chicks	Avg body wt 7 wk, g	% of body weight (avg)				
			Eye*	Thyroid†	Heart	Liver and bile sac	Spleen
A	6	532	0.343 (0.289-0.396)	0.008	0.503	2.776	0.210
B	9	399	0.638 (0.565-0.762)	0.007	0.537	2.893	0.232

Diet A. 113GN + 5% gelatin, 10 mg % nicotinic acid (control group).

Diet B. 113GN + 5% gelatin, 10 mg % nicotinic acid, 8% glycine.

* Values given are for one eye.

† Values given are for both thyroids.

basis as were the eyes from the controls. This striking eye condition may be seen in Fig. 1 which shows the difference in size of 2 excised eyeballs, one from a control chick and the other from a chick which received 8% of glycine.

The production of an apparent overgrowth of the eyeballs seems to be related to the form in which the amino acid is fed. The same basal diet supplemented with 10% of gelatin, 25% of bone ossein, and 10 mg of nicotinic acid per 100 g did not cause an enlargement of the eyeballs, tremors, or marked growth depression, although this diet contained approximately 8% of glycine but in the peptide form. (Bone ossein was used in place of gelatin because the diet tended to stick to the beaks of chicks when it contained more than 10% of gelatin. It is assumed that bone ossein and gelatin are equally digestible because both depressed growth in about the same order of magnitude when fed to chicks receiving nicotinic acid-low diets. No explanation for the protective action of protein is offered.)

Furthermore, the effect of free glycine under the conditions of these experiments is confined to the growing chick. In experiments

with laying hens, 4, 8, and 12% of glycine in a semi-purified diet containing 50 mg of nicotinic acid per 100 g did not cause any eye abnormalities during successive feeding periods of 5, 3, and 4 weeks, respectively. Feed consumption and egg production were only slightly lowered during the latter 4-week treatment.

The underlying physiological cause of the observed biological anomaly is as yet unknown. Whether the enlargement of the eyeballs is the result of actual tissue growth, or increased optic fluid caused by disturbed osmotic relationships, or other conditions must be determined through additional experimentation. The fact that the condition was observed in young growing chickens and not in laying hens would lend support to the belief that the enlargement is the result of actual overgrowth of tissue and not to increased ocular fluid caused by osmotic disturbances. Whether or not other amino acids will cause this same condition has not been studied.

Summary. Growth depression and tremors were observed in chicks fed 8% of glycine as part of a highly purified diet. The depressed growth rate was not overcome by feeding unusually high levels of nicotinic acid. In addi-



FIG. 1.

Contrast in size of eyeballs. The eyeball on the left is from a 7-week-old chick which received a diet containing 8% of glycine; its body weight was 395 g. The eyeball on the right is from a normal 7-week-old chick; its body weight was 574 g.

tion, a peculiar enlargement of the eyeballs developed which interfered with normal

vision and functioning of the nictitating membrane. Eight percent of glycine as the peptide produced no such effect. The action of glycine appeared to be confined to the growing chicken. The feeding of 12% of glycine to hens over an extended period produced no apparent ocular abnormalities.

We are indebted to Merck & Co., Rahway, N. J., for crystalline vitamins; Wilson and Co., Inc., Chicago, Ill., for 2X gelatin; Allied Mills, Inc., Portsmouth, Va., for soybean oil; Abbott Laboratories, North Chicago, Ill., for haliver oil; U. S. Industrial Chemicals, Inc., Baltimore, Md., for DL-methionine; and to Lederle Laboratories, Pearl River, N. Y., for pteroylglutamic acid.

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Effect of Dicumarol on Ac-Globulin and Prothrombin Activity.*

JOHN L. FAHEY, JOHN H. OLWIN, AND ARNOLD G. WARE.
(Introduced by Walter H. Seegers.)

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The administration of dicumarol to prevent the occurrence or extension of intravascular thromboses has given encouraging results.¹⁻³ The rationale of this treatment is based upon the ability of dicumarol to lower the prothrombin concentration in the circulating plasma.³ Dicumarol has been thought to act principally upon the prothrombin portion of the coagulation mechanism and thus to depress the clotting activity of whole blood. It is now apparent that another factor, Ac-globulin, warrants study in connection with dicumarol therapy. Ac-globulin⁴ is a plasma pro-

tein normally found in the circulating plasma. It acts to assure normal physiological conversion of prothrombin to thrombin during the process of blood coagulation. A deficiency of this factor impairs thrombin formation and a bleeding tendency may develop.⁵

The existence of Ac-globulin was unknown until recently, and previous reports on prothrombin measurements by both the one- and two-stage tests have not considered the possible role of this factor. With accurate and specific methods now available, a study was undertaken of both prothrombin and Ac-globulin levels as they are influenced by dicumarol administration.

Methods. Blood was obtained by venipuncture. In order to reduce contamination by tissue fluids the initial 3 cc of blood drawn into the syringe were discarded. The sample to be analyzed was received into a second syringe containing the anticoagulant. In this

* Aided by grants from the United States Public Health Service and the Ortho S. A. Sprague Memorial Institute Fund.

1 Olwin, J. H., Josiah Macy, Jr., Conference on Blood Clotting and Allied Problems, N. Y., 1948.

2 Allen, E. V., Hines, E. A., Kvale, W. F., and Barker, N. W., *Ann. Int. Med.*, 1947, **27**, 371.

3 Link, K. P., *Harvey Lectures*, 1943-44, 162.

4 Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, **172**, 699.

5 Owren, P. A., *Lancet*, 1947, **252**, 446.

manner 9 volumes of blood were added to 1 volume of 3.2% sodium citrate. After centrifugation at 3,000 rpm for 30 minutes the plasma was carefully removed from the cellular elements and frozen at -20°C until analyzed. Quantitative prothrombin determinations were carried out by means of a modified 2-stage test in which Ac-globulin is supplied.⁶ By adding an excess of accelerator in this modification, maximum conversion of prothrombin to thrombin is assured under the conditions specified in the 2-stage prothrombin analysis.⁷

Ac-globulin was measured by a method in which the concentrations of prothrombin, thromboplastin, and other known variables are controlled with the result that the rate of thrombin formation is dependent upon the amount of Ac-globulin present.¹ Because this test for Ac-globulin is a measure of a reaction rate (prothrombin to thrombin) and quite sensitive to minor changes in the reacting medium, all determinations were related to a control plasma of the same species. The normal control plasmas were carefully collected and frozen at -20°C until they were measured at the same time as the unknown samples.

Experimental. 1. Canine species. Seven normal healthy dogs were selected and the normal levels of plasma prothrombin and Ac-globulin determined. On successive days dicumarol was administered orally either as 2 doses of 14 mg per kg of body weight or as 4 doses of 4 mg per kg. Samples of blood for analysis were obtained regularly either from the saphenous or antecubital vein.

The results of these experiments indicate a sharp drop in plasma prothrombin which is maintained for a period of about a week. Following this a gradual restoration of prothrombin activity takes place. In addition, there was generally an initial decrease in Ac-globulin activity upon dicumarolization, though the magnitude of fall was much less than that of the prothrombin. This reduction

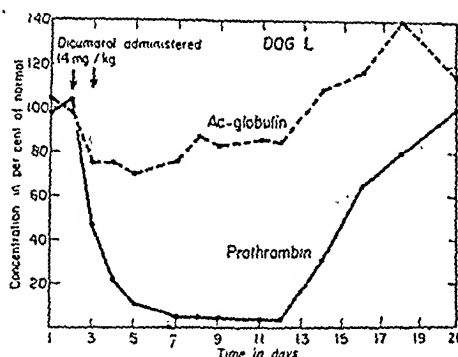


FIG. 1.

Changes in plasma Ac-globulin and prothrombin concentrations from dicumarol administration to a dog.

in Ac-globulin is only moderate, rarely falling under 65% of normal, and is in contrast to the severe fall in the prothrombin concentration. Even with the larger doses of dicumarol the Ac-globulin level did not fall below 50% of normal as compared to prothrombin titers reduced to as low as 1% of normal. In Fig. 1 are illustrated the results of a typical experiment employing a 9.0 kg female dog.

Considerable individual variation in the depth and duration of the response of Ac-globulin was observed. The drop generally began on the day following the first dose of the drug but on one occasion it did not occur until the 4th day. Recovery usually followed in 10-14 days. Interestingly, the increase in Ac-globulin concentration extended to values above the original levels, in one instance going as high as 150%, before returning to the normal range. This rise generally coincided with the period of marked prothrombin restoration. The dosage of 4 mg per kg of body weight approximates the original dose employed clinically in dicumarol therapy, though a total of 4 such doses is seldom utilized. In order to achieve maximal effects, 2 doses of 14 mg per kg were given to 5 of the dogs.

The prothrombin response to dicumarol in all dogs studied followed a pattern similar to that shown in Fig. 1, with the maximal reduction varying from 6% to less than 1% of normal. Both dosage schedules resulted in marked reductions of prothrombin. The smaller doses given over a greater period of time usually produced a slightly longer de-

⁶ Seegers, W. H., and Ware, A. G., Josiah Macy, Jr., Conference on Blood Clotting and Allied Problems, N. Y., 1948.

⁷ Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, 66, 801.

pression of Ac-globulin.

2. *Human Patients.* Preliminary investigations were carried out on 8 patients to whom dicumarol was administered after the occurrence of intravascular thromboses. Prothrombin values were reduced under this therapeutic regime and maintained at the desired low levels. Whenever possible, samples for analysis were obtained from the patients before dicumarol therapy was instituted. The prothrombin and Ac-globulin levels were followed as systematically as possible in each case, usually at 2- or 3-day intervals.

These studies indicate that in the human being as well as in the experimental animal the Ac-globulin level is lowered following the first doses of dicumarol. The concentration may be reduced to half of that normally found in human beings. Some individual variation was observed. As the prothrombin concentration was brought to the desired level of about 20% and the dicumarol doses reduced to maintain it so, the Ac-globulin titer returned to normal values. This restoration was found to be complete after about 3 weeks of therapy. Even when followed into the fourth week of dicumarol administration none of these patients showed a concentration of Ac-globulin above normal. The prothrombin and Ac-globulin levels of a patient, recorded in Table I, are representative of our general experience.

In addition to those patients followed from a time prior to or early in therapy, plasma samples were also analyzed from 12 out-patients who had received dicumarol for periods

ranging from 1 to 14 months. Prothrombin levels had been reduced as rapidly as was safely possible and for the most part were maintained within a range of between 15 and 30% of normal. These patients who had received dicumarol for long periods of time showed no appreciable variation in Ac-globulin from normal levels.

Discussion. These observations in general agree with those of Owen and Bollman⁸ who report on the effect of dicumarol in dogs. However, those authors do suggest a disappearance of the accelerator in the early stages of dicumarolization whereas no such marked alteration was found by us. The difference between the marked fall in accelerator indicated by their work and the relatively small decreases in Ac-globulin reported in this present study, even though higher dicumarol doses were utilized, may possibly be attributed to the methods of analysis used. The test developed in this laboratory is believed to be more sensitive to Ac-globulin and less sensitive to other factors in the plasma than is the method utilized by Owen and Bollman. The period of increased convertibility noted in their work in the recovery stages agrees with our observations which indicate that Ac-globulin concentrations in the canine species may reach 150% of the normal during that time.

Study of the Ac-globulin level in dogs is beset with certain problems which unless controlled may lead to erroneous results. The blood of a normal dog when removed from the vein clots rapidly. The Lee-White time of dog blood is 3-5 minutes compared to 6-9 minutes for normal human blood. If incipient initiation of the clotting process occurs in the sample to be tested the small amount of thrombin formed will partially convert the plasma Ac-globulin to the active form, serum Ac-globulin.⁹ Experience has shown that this effect is easier to avoid when citrate is used as anticoagulant. The presence of a small amount of serum Ac-globulin will indicate an apparent but false increase in the plasma Ac-

TABLE I.

Plasma Ac-globulin and Prothrombin Levels in a Human Patient Receiving Continuous Dicumarol Therapy.

Days following initial administration of drug	Prothrombin % of normal	Ac-globulin % of normal
1	77	—
2	34	49
3	56	52
5	35	49
7	24	68
9	20	59
12	21	64
14	26	67
16	26	89

⁸ Owen, C. A., and Bollman, J. L., *Proc. Soc. Exp. Biol. and Med.*, 1948, 67, 231.

⁹ Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1948, 152, 567.

globulin present.⁹ On the other hand, if a considerable amount of thrombin is released prior to the effect of the anticoagulant it may actually destroy part of the Ac-globulin. To guard against these difficulties the procedure described in this paper for drawing blood was followed carefully. Sodium citrate is also preferable to oxalate as an anticoagulant because Ac-globulin is more stable in stored citrated dog plasma than in oxalated plasma.

It is certainly possible, or even probable, that the effect of dicumarol on the coagulation mechanism is not restricted to prothrombin and Ac-globulin alone. Conflicting reports^{10,11} have been presented as to the susceptibility of fibrinogen to dicumarol and a variation in serum antithrombin titer has also been proposed.¹² From the observations reported here it is seen that dicumarol has, in addition to its paramount function of lower-

ing the prothrombin concentration, a secondary effect upon the coagulation mechanism through its reduction of Ac-globulin early in the period of dicumarol therapy. The definite drop in Ac-globulin which occurs following initiation of therapy should contribute at that time to the prevention of clot formation.

Summary. In human patients receiving dicumarol the plasma Ac-globulin level may be depressed by 20-50% following initiation of therapy. Individual variation was noteworthy. A gradual return to normal concentrations of Ac-globulin occurs within 3 weeks as therapy is continued and the prothrombin is maintained at a low titer. No appreciable difference from normal was found in the Ac-globulin values of patients who had been on dicumarol therapy for 1 to 14 months.

Dogs receiving larger dicumarol doses than were administered to human patients showed a similar Ac-globulin response and a more marked reduction in prothrombin. A period of slightly lowered Ac-globulin activity in the dog is followed by a temporary rise to levels above normal.

¹⁰ Irish, U. D., and Jaques, L. B., *Am. J. Physiol.*, 1945, **143**, 101.

¹¹ Peters, H. R., Doenges, J. P., and Brambel, C. E., *Southern Med. J.*, 1948, **41**, 526.

¹² Hurn, M., Barker, N. W., and Mann, F. D., *Am. J. Clin. Path.*, 1947, **17**, 712.

16766

Indoleacetic Acid Studies in Man.*

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Corn products have usually been present in diets which have been associated with the etiology of pellagra in man,¹ blacktongue in

dogs,² and nicotinic acid deficiency in rats.³ It has been shown that the role of corn in the development of these deficiency syndromes is not due only to the low nicotinic acid content of the corn.^{2,4,5} Other factors may be

* A preliminary report was presented at the meeting of the American Society of Biological Chemists, March, 1947. (*Fed. Proc.*, 1947, **6**, 288).

This work was aided by grants from the Nutrition Foundation, Inc., the Williams Waterman Fund of the Research Corporation, and the United States Public Health Service.

¹ Frazier, E. L., and Friedemann, T. E., *Quart. Bull. Northwestern Univ. Med. School*, 1946, **20**, 24.

² Handler, P., *Proc. Soc. Exp. Biol. and Med.*, 1943, **32**, 263.

³ Krehl, W. A., Teply, L. J., and Elvehjem, C. A., *Science*, 1945, **101**, 283.

⁴ Aykroyd, W. R., and Swaminathan, M., *Indian J. Med. Res.*, 1940, **27**, 667.

⁵ Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, 1945, **101**, 489.

the low tryptophane content^{5,6,7} and the possible presence of a "pellagragenic" agent^{8,9} which could inhibit the utilization of nicotinic acid or the conversion of tryptophane to nicotinic acid.

The experiments on rats by Kodicek, Carpenter, and Harris⁹ in 1946 suggested that the growth depressing or "pellagragenic" effect of corn may be due to its content of indole-3-acetic acid. In the present experiments the effect of indoleacetic acid supplements on nicotinic acid and tryptophane metabolism in man was evaluated by a study of the urinary excretion levels of nicotinic acid, tryptophane, and related metabolites, by subjects maintained on a controlled diet. While this work was in progress, other laboratories reported that indoleacetic acid had no effect on the growth of rats¹⁰⁻¹² or on the excretion of N'-methylnicotinamide (N'-Me) by rats.¹² Kodicek, Carpenter, and Harris¹³ have also been unable to duplicate their earlier results with indoleacetic acid.

Experimental. The subjects were ward patients who were found to be free of complicating organic disease as determined by clinical and laboratory examinations. They were maintained in a separate metabolism ward and were permitted to be ambulatory. The wheat diet, which was used to maintain these subjects has been employed in other nicotinic acid and tryptophane studies.^{7,14} This diet, which was planned to provide 2400 calories, contained 248 g of unenriched wheat

products and supplied 43 g of protein, 430 mg of tryptophane, and 6 mg of nicotinic acid per day. Only 10% of the total protein was of animal origin. Variations in food intake, which were necessary in order to adjust the caloric intake, were recorded. After suitable control periods on these diets, indole-3-acetic acid (Eastman) was added as a supplement with dinner and supper.

Twenty-four-hour urines were collected in amber bottles containing 15 ml of glacial acetic acid, stored in the refrigerator and pooled in 48-hour periods for analysis of creatinine, nitrogen, thiamine, riboflavin, nicotinic acid, N'-Me, and tryptophane by methods previously described.¹⁴ Since it has been observed that in rats and humans who had received tryptophane supplements, the apparent nicotinic acid content of the urine was markedly increased by autoclaving with strong acid,^{14,15} the urines in two of the present experiments were autoclaved in the presence of 1 N H₂SO₄ for 30 minutes prior to assay of nicotinic acid. Another metabolite, which appeared in the urine of subjects who had received extra tryptophane, has tryptophane-like activity for *L. arabinosus* but is extractable from urine (at pH 4) by ether.¹⁴ Ether extraction was tried in a few of the present experiments in an effort to detect any increase in this metabolite after indoleacetic acid administration.

Indoleacetic acid was estimated only in those urines obtained after administration of this substance, using the method suggested by Tang and Bonner.¹⁶ In this test tryptophane gave 0.7% of the color obtained with indoleacetic acid while indole produced 14% and xanthurenic acid 1.3% of the indoleacetic acid color. About 10 to 15 mg of indoleacetic acid per day was found by this method in urines collected from the subjects during the basal period. Since many indole derivatives which appear in urine may interfere with this test, these basal values were not considered to be reliable.

⁶ Singal, S. A., Sydenstricker, V. P., and Littlejohn, J., *Fed. Proc.*, 1947, **6**, 422.

⁷ Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, 1947, **167**, 293.

⁸ Woolley, D. W., *J. Biol. Chem.*, 1946, **163**, 773.

⁹ Kodicek, E., Carpenter, K. J., and Harris, L. J., *Lancet*, 1946, **2**, 491.

¹⁰ Krehl, W. A., Henderson, L. M., de la Huerga, J., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, **166**, 531.

¹¹ Krehl, W. A., Carvalho, A., and Cowgill, G. R., *Fed. Proc.*, 1947, **6**, 413.

¹² Rosen, F., and Perlzweig, W. A., *Arch. Biochem.*, 1947, **15**, 111.

¹³ Kodicek, E., Carpenter, K. J., and Harris, L. J., *Lancet*, 1947, **2**, 616.

¹⁴ Sarett, H. P., and Goldsmith, G. A., *J. Biol. Chem.*, in press.

¹⁵ Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, 1946, **166**, 573.

¹⁶ Tang, Y. W., and Bonner, J., *Arch. Biochem.*, 1947, **13**, 11.

TABLE I.
Effect of Indoleacetic Acid Administration on Daily Excretion of Tryptophane, Nicotinic Acid, and Related Compounds by Subject I.

Diet per day	Days	Creatinine, g	Nitrogen, g	Thiamine, γ	Riboflavin, γ	Nicotinic acid, mg	N'-Me, mg	Tryptophane, mg
Wheat (2520 calories, 44 g protein)	1-2	1.1	5.9	71	470	0.5	1.6	11
	3-4	1.1	5.6	44	235	0.4	1.2	11
	5-6	1.3	5.4	50	150	0.5	1.4	13
	7-8	1.2	5.0	30	115	0.4	1.3	12
Wheat + 25 mg indoleacetic acid	9-10	1.3	5.8	33	107	0.5	1.6	13
	11-12	1.3	5.9	29	104	0.5	1.6	13
	13-14	1.2	5.7	20	53	0.5	1.2	14
	15-16	1.2	5.7	22	52	0.5	1.1	13
Wheat + 50 mg indoleacetic acid	17-18	1.2	5.8	21	53	0.5	1.5	14
	19-20	1.1	5.2	20	56	0.4	1.3	12
	21-22	1.2	5.3	19	65	0.5	1.2	15
	23-24	1.2	5.6	25	—	0.6	1.3	16
Wheat + 100 mg indoleacetic acid	25-26	—	—	—	—	—	—	—
	27-28	1.0	4.9	16	61	0.6	1.1	11
	29-30	1.1	5.5	21	80	0.6	1.3	15
	31-32	1.0	5.7	21	66	0.5	1.8	14
Wheat	33-34	1.1	5.8	27	74	0.5	1.8	15

TABLE II.
Effect of 200 mg of Indoleacetic Acid on Daily Excretion of Tryptophane, Nicotinic Acid, and Related Compounds by Subject I.

Effect of 200 mg of Indoleacetic Acid on Daily Excretion of Tryptophan, Nicotinic Acid, and Acetic Compounds by Rats							
Diet per day	Days	Nicotinic acid		N'-Me, mg	Tryptophano		Indoleacetic acid, mg
		As is, mg	Acid hydrol., mg		As is, mg	After ether extr., mg	
Wheat (2530 calories, 44 g protein)	1-2	0.7	1.1	2.3	16		
	3-4	0.5	0.8	1.3	12		
	5-6	0.5	1.1	1.5	13		
	7-8	0.5	1.0	1.2	13	12	
Wheat + 200 mg indoleacetic acid	9-10	0.7	1.4	1.3	18	17	130
	11-12	0.6	1.2	1.4	18	17	138
	13-14	0.5	1.2	1.4	17		
	15-16	0.6	1.7	1.9	16		83
Wheat	17-18	0.7	1.8	1.0	13		
	19-20	0.5	0.9	1.4	12		
	21-22	0.6	1.2	1.2	15		

TABLE III.
Effect of 200 mg of Indoleacetic Acid on Daily Excretion of Tryptophane, Nicotinic Acid, and Related Compounds by Subject II.

Effect of 200 mg of indoleacetic acid on daily excretion of tryptophane, nicotinic acid, and indoleacetic acid									
Diet per day	Days	Nicotinic acid			N'-Me, mg	Tryptophane		Indoleacetic acid, mg	
		As is, mg	Acid hydrol., mg	As is, mg		After ether extr., mg			
Wheat (2370 calories, 41 g protein)	1-2	0.6	1.2	2.7	6				
	3-4	0.4	0.9	1.7	4				
	5-6	0.6	1.3	2.4	5				
	7-8	0.5	1.2	2.2	5	4			
Wheat + 200 mg indoleacetic acid	9-10	0.6	1.3	2.1	6	5		70	
	11-12	0.5	1.2	2.1	6	5		97	
	13-14	0.6	1.3	1.8	6			73	
	15-16	—	—	—	—			—	
Wheat	17-18	0.5	1.5	1.3	4				
	19-20	0.5	1.1	1.6	4				
	21-22	0.5	1.2	1.5	5				

Results. The values obtained in the analyses of the urine in these experiments are presented in Tables I, II and III. The subject for the experiments shown in Table I, and III was a 30-yr.-old male (W.H.) and in Table II a 47-yr.-old female (J.H.) The subjects were maintained on the basal-wheat diet for 8 days before the supplements of 25 to 200 mg of indoleacetic acid were given. Since corn is reported to contain 20 to 100 mg of indoleacetic acid per kg^{17,18} 25 mg of indoleacetic acid represents that found in 0.25 to 1.2 kg of corn while 200 mg of indoleacetic acid is equivalent to that obtained from 2 to 10 kg of corn products.

The creatinine and nitrogen excretion remained fairly constant in each experiment and are an indication of the completeness of urine collection and the constancy of the diet. The levels of thiamine and riboflavin excreted decreased rapidly, as has been found with other subjects on this diet,¹⁴ and were not affected by the indoleacetic acid administration. For the sake of brevity, the above findings are shown only for the experiment in Table I.

The urinary nicotinic acid values obtained by microbiological analysis of the diluted urine were not significantly changed throughout the course of the experiments. The nicotinic acid values which were obtained after autoclaving the urine with acid (Tables II and III) also remained relatively constant. The slight increase in excretion of this metabolite at the end of the period of indoleacetic acid administration (Table II) is of questionable significance. Tryptophane is the only known compound which, when added to the diet, leads to an increase in excretion of this acid-hydrolyzable nicotinic acid-like compound.^{14,15}

The excretion of N'-Me is at present the main index of nicotinic acid metabolism. In the present experiments there were no significant changes in excretion of N'-Me after administration of indoleacetic acid. In previous studies it has been shown that replace-

ment of 190 g of the wheat of this diet by corn products resulted in a marked decrease in excretion of N'-Me.^{7,14} This may have been due to the presence of an inhibitor in the corn,⁸ or to the lower tryptophane content of the corn as compared with the wheat, or to a combination of these factors.

Tryptophane excretion increased slightly in all 3 experiments following the administration of indoleacetic acid and decreased to approximately the basal level after the supplementation was discontinued. The presence of indoleacetic acid in the urine could not account for the increased tryptophane values. Relatively high levels of indoleacetic acid (0.1 mg/ml) in the microbiological assay have been shown to have a slight stimulatory effect upon the utilization of tryptophane by *L. arabinosus*.¹⁹ However, urines which were diluted for analysis of tryptophane, supplied less than 50 γ of indoleacetic acid for each 10 ml assay tube. Experiments in this laboratory show that 20 of 500 γ of indoleacetic acid per 10 ml assay tube has no activity for *L. arabinosus* in a test which can detect less than 0.2 γ of tryptophane and has no significant effect on tryptophane utilization. However, the metabolism of indoleacetic acid, either in the intestine or in the body, may lead to the formation of other compounds which can replace tryptophane or stimulate the utilization of tryptophane by *L. arabinosus*. Only 70 to 138 mg of indoleacetic acid were found in the urine after oral administration of 200 mg of indoleacetic acid (Tables II and III).

Extraction of some of the urines with ether showed (Tables II and III) that the increase in tryptophane excretion after indoleacetic acid supplementation was not due to the ether extractable tryptophane-like substance which is found in the urine after tryptophane administration.¹⁴

Discussion. In the present experiments the addition of up to 200 mg of indoleacetic acid per day to a low protein diet appeared to have no significant effect on nicotinic acid utilization or on the conversion of trypto-

¹⁷ Haagen-Smit, A. J., Leech, W. D., and Bergren, W. R., *Am. J. Botany*, 1942, **29**, 500.

¹⁸ Berger, J., and Avery, G. S., *Am. J. Botany*, 1944, **31**, 199.

¹⁹ Handler, P., and Kamin, H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 251.

phane to nicotinic acid as evidenced by the excretion of nicotinic acid and N'-Me by man. Rosen and Perlzweig¹² showed that indoleacetic acid had no effect on the excretion of N'-Me by rats receiving a basal diet alone or with tryptophane supplements. Others have also been unable to obtain depression of rat growth by inclusion of indoleacetic acid in the diet, although the addition of corn or protein low in tryptophane has retarded growth.¹⁰⁻¹³ The replacement of a portion of the wheat diet by corn products has been shown to decrease the excretion of N'-Me by man.^{7,14} This effect may be due to the lower tryptophane content of the corn as compared with the wheat or to the presence of an unidentified inhibitory agent in the corn.⁸

The increase in excretion of tryptophane compounds by man after the ingestion of indoleacetic acid may be due to the conversion of indoleacetic acid to a substance which has

tryptophane-like activity for *L. arabinosus*. It is also possible that indoleacetic acid may have some effect on tryptophane metabolism in man which is separate from the tryptophane-nicotinic acid relationship. In rats, indoleacetic acid cannot replace tryptophane as a growth stimulant, when added to a diet deficient in tryptophane and nicotinic acid.¹⁰⁻¹³

Summary. The addition of 25 to 200 mg of indoleacetic acid per day to a diet low in protein and nicotinic acid appears to have no significant effect upon the utilization of tryptophane and nicotinic acid by man as evidenced by urinary excretion studies.

The author wishes to thank the following people for their cooperation in these studies: Drs. Grace A. Goldsmith and Roy E. Butler for clinical assistance, Janice Gibbens and Winifred Bouvet for planning and supervising the diets, and Antoinette Dingraudo, Carol Haas and Janice Loeb for their technical assistance.

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Changes in Circulating Eosinophils in Man Following Epinephrine, Insulin, and Surgical Operations.*

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Forsham and his associates¹ have reported that pituitary adrenocorticotrophic hormone (ACTH) produces a fall in circulating eosinophils which is dependent upon normal adrenal cortical function. Long and Fry² had previously demonstrated that epinephrine stimulates the adrenal cortex of the rat, and that this effect is mediated by the anterior pituitary. For these reasons we have studied the effects upon the circulating eosinophils of

epinephrine, and later of insulin and of surgical operations. Studies have been made upon healthy persons, patients with adrenal and pituitary insufficiency, and patients with "diseases of adaptation"³ such as essential hypertension and nonspecific colitis.

While this study was in progress, eosinopenia after intravenous epinephrine and insulin was reported by Godlowski,⁴ and Thorn and his associates⁵ reported that intravenous epinephrine reduced the eosinophil count in

* Aided by a generous gift from Mr. John L. Given.

¹ Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G., *J. Clin. Endocrinol.*, 1948, 8, 15.

² Long, C. N. H., and Fry, E. G., *Proc. Soc. Exp. Biol. and Med.*, 1945, 59, 67.

³ Selye, H., *J. Clin. Endocrinol.*, 1946, 6, 117.

⁴ Godlowski, Z. Z., *Brit. Med. J.*, 1948, 1, 46.

⁵ Recant, L., Forsham, P. H., and Thorn, G. W. Read at the thirtieth meeting of the Association for the Study of Internal Secretions, June 18, 1948.

Eosinophil Counts following Epinephrin, Insulin, Surgery

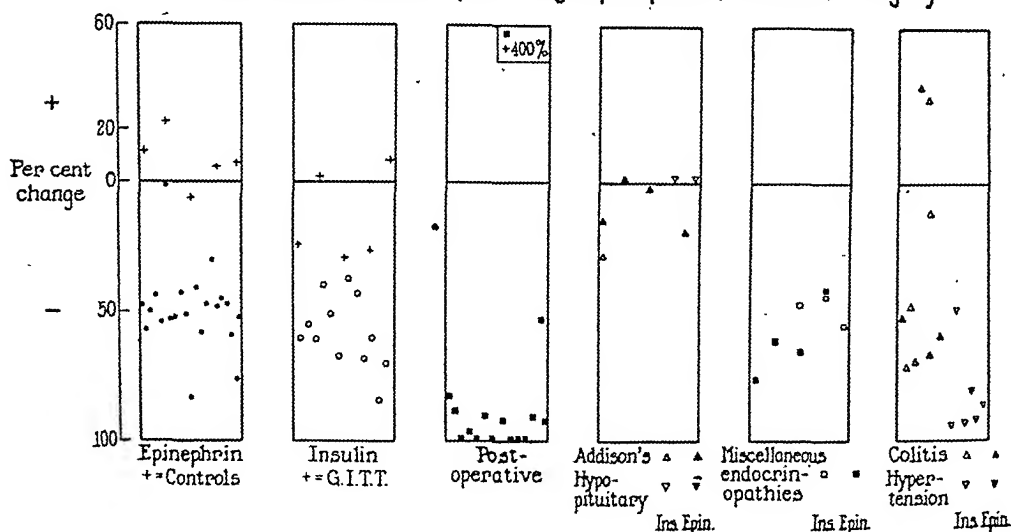


FIG. 1.

persons with normal adrenals. Gershberg and Long⁶ demonstrated that insulin stimulated the adrenal cortex of animals with intact pituitaries.

Method. The eosinophils of heparinized venous blood were enumerated in the Levy double depth counting chamber, using a modification of the method of Dunger.⁷

Results (see Figure). The normal range of eosinophil count was found to be 100-300 per cu mm.

Epinephrine. Epinephrine HCl, 0.5 cc of 1:1000 solution, was administered subcutaneously to 21 healthy subjects. In 20 of these subjects the eosinophil count dropped by an average of 56% in 2½ - 3 hours. Five subjects given 0.5 cc saline showed no significant drop in eosinophils.

Insulin. Regular insulin 0.1 unit/kg was given intravenously to 11 healthy subjects and to one patient with moderately advanced pulmonary tuberculosis who was on prolonged bed rest. In all these subjects the eosinophil count fell, the average change being minus 57.5%, four hours after injection. In all cases a blood sugar of 60 mg% or below was

attained. Five subjects were given standard glucose-insulin tolerance tests as controls. Two of these patients showed no significant change; the other 3 had slight drops (22 to 28%) but in these 3 we were unable to obtain neutral sugar curves.

Addison's Disease and Pituitary Insufficiency. In 4 cases of Addison's Disease and 2 cases of pituitary insufficiency the typical fall in eosinophil count after epinephrine and insulin did not develop. The largest change observed was a 28% fall, associated with sustained hypoglycemia in a patient with Addison's Disease.

Miscellaneous Endocrinopathies. In this group are 2 acromegals, 2 cases of pituitary tumor, 1 of Cushing's syndrome, and 1 of possible Cushing's. This group all responded normally with an average drop of 55.2% after epinephrine or insulin.

Hypertension. Six patients with essential hypertension have been studied. Four showed an exaggerated response to insulin with an average drop in eosinophils of 90.4%. Blood sugar curves were similar to those observed in the healthy group. A fifth mild hypertensive dropped 79% after epinephrine and the sixth patient showed a 48% drop after insulin.

Colitis. Of 3 patients with severe ulcera-

⁶ Gershberg, H., and Long, C. N. H., *ibid.*

⁷ Dunger, A., *Munchen. Med. Wchnschr.*, 1910, 57, 1942.

tive colitis given insulin, 2 showed a rise in eosinophils (32%, 37%) and a third only a 12% drop. Five patients with milder ulcerative colitis showed a normal response.

Surgical Operations. Fifteen patients have been followed through the stress of major surgery. Following operation, 14 of these patients showed profound drops in their eosinophil counts, 6 falling to zero. In general the degree of fall varied directly with the magnitude of the operation. The peak of response occurred most commonly 3-8 hours after the beginning of the operation. The fifteenth patient, one with severe ulcerative colitis, showed a marked and progressive eosinophilia following an ileostomy.

Discussion. Epinephrine and insulin appear to exert similar effects on the circulating eosinophils of man, and our results suggest that this effect is mediated through the pituitary and adrenal cortex. The failure of response in a small number of cases of Addison's Disease and pituitary insufficiency leads us to anticipate that the eosinophil changes following epinephrine and insulin may be useful in the diagnosis of these conditions.

Browne and his associates⁸ have measured

the changes in excretion of glycogenic corticoids and other indices of adrenal function following major surgery, and have interpreted these changes as part of the process of adaptation to non-specific stress. The occurrence of eosinopenia following operations may be construed as further evidence of the role of the adrenal cortex in the "alarm reaction" in man.

In view of the postulated role of the adrenal in the genesis of "essential" hypertension, the exaggerated eosinophil response in patients with this disorder is being studied further. The absence of an eosinophil response in three patients with severe chronic ulcerative colitis suggests that adrenal insufficiency may occur in such patients. This possibility is being investigated.

Summary. Significant eosinopenia has been observed following injection of epinephrine and of insulin. This change was exaggerated in patients with essential hypertension, and diminished or absent in patients with adrenal or pituitary insufficiency. Marked eosinopenia has been found after surgical operations.

⁸ Browne, J. S. L., Conference on Bone and Wound Healing, Josiah Macy, Jr., Foundation, December 11-12, 1942, pp. 45-47.

The authors are indebted to Dr. Peter Forsham for technical advice.

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Electrophoretic Patterns After Dicumarol Medication.

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Dicumarol induces prothrombinopenia by inhibiting the production of prothrombin, presumably in the liver. At dosage levels used for therapeutic purposes, its action is reversible and can be counteracted by vitamin K.¹⁻³ In order to determine whether or not protein components other than prothrombin were measurably altered by the drug, comparisons by means of electrophoresis of

human plasma before and after Dicumarol medication were made.

¹ Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 1.

² Shapiro, S., Redish, M. H., and Campbell, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 12.

³ Cromer, H. E., Jr., and Barker, N. W., *Proc. Staff Meet. Mayo Clin.*, May, 1944, **19**, 217.

TABLE I.
Electrophoretic Data and Prothrombin Times on Plasma from Patients after Dicumarol.

Specimen	% concentration of					A/G	Prothrombin time
	Albumin	Globulins					Whole plasma, sec.
		α	β	Φ	γ		
Ma I	57.8	8.5	10.0	8.2	15.4	1.37	17.5
Ma II	59.3	8.2	11.9	6.7	13.9	1.45	39.5
Mo I	66.8	7.2	14.2	3.5	8.5	2.00	16.0
Mo II	66.2	7.6	13.9	3.8	8.3	1.97	40.6
Ox I	65.1	5.7	11.4	4.5	13.3	1.86	15.0
Ox II	64.4	4.5	12.2	4.5	14.4	1.81	38
Ow I	45.9	11.6	18.0	9.1	15.2	0.85	15.2
Ow II	42.8	12.0	17.3	9.1	18.8	0.76	23.0
Me I	49.7	8.6	21.0	6.9	13.8	0.99	15.2
Me II	49.7	6.6	21.8	9.1	12.8	0.98	37.6
Kr I	54.1	9.9	13.4	5.3	17.4	1.18	13.8
Kr II	56.2	9.2	12.5	4.5	17.6	1.28	23.5

Mo and Ox = Normal subjects.

I = Before medication (dicumarol).

II = 48 hr later.

Material. The plasmas of 6 adult persons, 3 of each sex, were studied. Four were patients being treated for intravascular thrombosis, and 2 were normal subjects. The doses of Dicumarol used in each case varied between 500 and 700 mg given in 2 doses about 12 hours apart, the first dose being usually about 100 mg greater than the second. Prothrombin estimations were made immediately prior to the administration of the initial dose and again about 48 hours later. Samples of blood for electrophoresis were obtained at the same time.

Estimations of prothrombin time were made by a method previously described.⁴

Since hemoglobin migrates electrophoretically with γ -globulin, hemolysis was carefully avoided. Plasmas from oxalated blood samples were diluted with 2 volumes of 0.02M sodium phosphate buffer of pH 7.4 which was 0.15M with respect to NaCl, ionic strength 0.2, and dialyzed in the cold against the same buffer solution for one day. Analyses were made in the Tiselius apparatus in the usual manner.

Results. Planimeter measurements and

the calculated percentages of the several plasma components are recorded in Table I. It is apparent from this table, that no consistent changes occurred in the electrophoretic patterns although there was marked Dicumarol-induced prothrombinopenia. This was true for samples of plasma taken both for 48 and 72 hours after Dicumarol administration. The quantity of prothrombin in plasma is too small to be demonstrated by electrophoresis, therefore, no evidence of its depression was expected in the patterns. Only one patient, Kr, exhibited a significant change in A/G ratio. Before medication, the value of this ratio was 1.18. It rose to 1.28 after 48 hours and dropped to 0.95 when estimated 24 hours later. The fall was a reflection of a change in all components. This patient had generalized arteriosclerosis, circulatory failure and peripheral venous thrombosis. It is possible that the disease processes were responsible for this change. We hesitate to ascribe it to the influence of the drug.

Summary. Dicumarol at therapeutic dosage levels failed to produce alterations in human plasma of sufficient magnitude to be detected by electrophoretic analysis.

⁴ Shapiro, S., Sherwin, B., Redish, M., and Campbell, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1942, 50, 85.

Comparison of New Jersey and Palestine Strains of Bovine Leptospira.

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In the previous work on leptospirosis in cattle in New Jersey^{1,2} the similarity to the disease reported in Palestine³⁻⁵ was obvious, but no culture of the Palestine strain was at hand for serological comparisons. The present paper reports serological studies carried out with 6 strains of leptospira isolated in an outbreak of the disease in a New Jersey dairy herd and a strain of bovine leptospira from Palestine.

Methods. Chang's⁶ semisolid and fluid media (in which rabbit serum had been substituted for horse serum and the hemoglobin omitted) were used for storing cultures and Schueffner's⁷ medium was used for the serological tests. Actively growing cultures incubated 5 to 7 days at 30°C were used as antigen either as living or formalinized preparations. Equal amounts of serum dilutions and leptospira suspensions were mixed in small test tubes. For agglutination tests formalinized cultures (0.3% formalin) were

incubated for 2 hours at 37° C and kept at room temperature, while with living antigen the incubation period was the same but the tubes were stored in the refrigerator. Readings were made the following morning by examining uncovered drops by dark-field illumination with objective 10 and ocular 10. The results with living cultures were recorded as +, all organisms lysed; ± many organisms lysed; and —, no lysis. With formalinized material the readings were recorded as +, nearly all leptospires agglutinated in large clumps; ±, many leptospires agglutinated in small clumps; and —, no agglutination.

Serological Examination. In the serological studies it was found that both living and formalinized cultures gave identical titers as a rule, yet it was useful to check the results of one method with the other. Small antibody concentrations may not be detected if living cultures are employed, as minor degrees of lysis are more difficult to read than slight agglutination reactions obtained with formalinized material. The disadvantage, however, of using live cultures is offset by the avoidance of spontaneous clumping which not infrequently occurs in formalinized cultures.

Three leptospiral strains (N.J.) were used to immunize rabbits. The rabbits received 3 intravenous injections of living cultures at weekly intervals and were bled one week after the last injection. Each New Jersey immune serum agglutinated the 3 New Jersey strains to the same titer. It was therefore assumed that the strains were identical and this was confirmed in absorption experiments. Subsequently 2 rabbits were immunized, one with a New Jersey culture, the other with the Palestine strain. Each animal received 4 injections of living culture intravenously and a week after the last injection they were bled from the heart. The results for both agglutination and

* Visiting investigator from the Department of Hygiene and Bacteriology, The Hebrew University, Jerusalem.

¹ Baker, J. A., and Little, R. B. Presented in abstract form before the Twenty-eighth Conference of Research Workers in Animal Diseases in North America, Chicago, Ill., Dec. 2, 1947. Also in *Bovine Mastitis. A Symposium* (R. B. Little and W. N. Platridge, editors), New York, McGraw-Hill Book Co., Inc., 1946.

² Baker, J. A., and Little, R. B., *J. Exp. Med.*, 1948, 88, 295.

³ Bernkopf, H., Olitzki, L., and Stuczynski, L. A., *J. Infect. Dis.*, 1947, 80, 53.

⁴ Ungar, H., and Bernkopf, H., *Arch. Path.*, 1947, 44, 59.

⁵ Bernkopf, H., Stuczynski, L. A., Gottlieb, T., and Halvay-Katz, C., *J. Infect. Dis.*, 1948, in press.

⁶ Chang, Shih Lu, *J. Infect. Dis.*, 1947, 81, 28.

⁷ Kelsner, R. A., and Schoening, H. W., *Manual of Veterinary Bacteriology*, 4th Edition, Williams and Wilkins Co., Baltimore, Md., 1943, p. 411.

TABLE I.
Agglutination and Lysis Tests with Immune Sera Prepared in Rabbits Against the New Jersey and the Palestine Strains of Leptospira.

Strain	Antigen used	Antiserum to New Jersey strain					Antiserum to Palestine strain				
		1:20	1:500	1:2,000	1:50,000	1:200,000	1:20	1:200	1:2,000	1:50,000	1:200,000
New Jersey	Formalinized culture	+	+	+	+	—	—	—	—	—	—
	Living culture	+	+	+	+	—	—	—	—	—	—
Palestine	Formalinized culture	++	—	—	—	—	++	++	++	++	—
	Living culture	++	—	—	—	—	++	++	++	++	—
<i>L. icterohaemorrhagiae</i>	Formalinized culture	—	—	—	—	—	—	—	—	—	—
	Living culture	—	—	—	—	—	—	—	—	—	—
<i>L. canicola</i>	Formalinized culture	—	—	—	—	—	—	—	—	—	—
	Living culture	—	—	—	—	—	—	—	—	—	—

TABLE II.
Examination of Sera of Two New Jersey Cows* for Antibodies Against the New Jersey and Palestine Strains of Leptospira.

Cow No.	Date of onset of illness	Date of bleeding	Agglutination of formalinized culture					Lysis of living culture				
			New Jersey strain		Palestine strain			New Jersey strain		Palestine strain		
			Dilution of sera		Dilution of sera			Dilution of sera		Dilution of sera		
1	12/2/46	12/2/46	1:20	1:200	1:20,000	1:50	1:200	1:20	1:200,000	1:20	1:200,000	1:20,000
2	4/6/48	1/9/47 4/6/48 4/26/48	+	+	+	+	+	+	+	+	+	+

* Bled at the onset of the disease and after recovery.

TABLE III.
Inoculation of the New Jersey Strain into Guinea Pigs Previously Inoculated with the Palestine Strain.

Guinea pig No.	First inoculation	Results of second inoculation with New Jersey strain
1	Palestine strain	Fever after 3 days
2	"	" " 6 "
3	"	" " 7 "
4	"	" " 7 "
5	"	No fever
6	"	" "
7	"	" "
8	Not inoculated	Fever after 2 days
9	" "	" " 3 "
10	" "	" " 3 "
11	" "	" " 3 "

lysis tests are given in Table I. Strains of *Leptospira canicola* and *Leptospira icterohaemorrhagiae* kindly supplied by Dr. R. E. Kelser were also included in this test.

It can be seen in Table I that no marked cross reactions occurred with the New Jersey and Palestine strains. Antiserum to the Palestine strain did not cross react with the New Jersey strain, while the antiserum to the New Jersey strain showed a weak cross reaction with the Palestine strain in a dilution of 1:20 (lysis) and 1:200 (agglutination). It is also clearly shown in Table I that serologically both bovine strains are different from *L. icterohaemorrhagiae* and *L. canicola*.

Examination of sera from naturally infected cows. Sera from cows of the New Jersey herd which had recovered from an attack of the disease reacted strongly with the New Jersey strain^{1,2} but showed little if any reaction against the Palestine culture.³ The reaction of the sera of 2 cows taken at the onset of the disease and 20 and 38 days later, respectively, is given in Table II. The specificity of the reaction with the New Jersey strain is clearly shown, for neither serum from the recovered animals agglutinated the Palestine culture.

The sera of 10 cows which had shown typical symptoms of the disease in May 1946 (bled 2 to 3 weeks after the onset of the illness) reacted with the New Jersey culture in dilutions up to 1:20,000. One serum agglutinated the Palestine strain in a dilution of 1:2,000. Of the bovine sera tested from both normal and recovered cases in the New Jersey herd, this was the only serum that agglutin-

ated the Palestine culture in any dilution.

Forty-six sera from cows in a barn in which 2 typical cases of abnormal milk (bloody or thick off-colored milk) had occurred 2 months earlier were tested against both strains. Eight of the sera gave a positive reaction with the New Jersey strain in a dilution of 1:200, and 4 in a 1:2,000 dilution, thus suggesting that inapparent infections are not uncommon.

Pathogenicity of the Strains for Guinea Pigs. A cross infection experiment was set up by inoculating guinea pigs first with the Palestine strain and 3 weeks later with a New Jersey strain isolated from the milk of cow No. 1489. Each animal received intraperitoneally one ml of a leptospira culture in fluid medium. The results are given in Table III.

The guinea pigs showed no reaction following the injection of the Palestine strain, confirming earlier observations.³ The New Jersey strain, however, produced fever in all control animals 2 to 3 days after the inoculation.² Only one of the 7 guinea pigs, which had received the Palestine strain before, developed fever 3 days after inoculation with the New Jersey strain. Three guinea pigs of the same group became febrile after an incubation period of 6 to 7 days (nearly twice as long as in the controls) while the remaining animals appeared normal during the entire period of observation. It is obvious therefore that, based on the development of fever, infection with the Palestine strain had produced complete immunity in 3 guinea pigs and partial immunity in 3 other animals out of a total of 7 tested.

A comparison of Tables I and III shows that the serological examination, because it is quantitative, gave a better differentiation of the strains. Schueffner and Mochtar⁸ found in numerous experiments that guinea pigs that had recovered from infection with one strain of leptospira were protected against strains which serologically were unrelated.

⁸ Schueffner, W., and Mochtar, A., *Z. f. Bakt. Abt. 1, Orig.*, 1926-27, 101, 405.

Summary. A study has been made of strains of leptospira recovered from an outbreak of leptospirosis among cattle in New Jersey and a strain isolated from an outbreak of the disease in Palestine. Sera from recovered cattle as well as sera from immunized rabbits were used in agglutination and lysis tests and it was found that all the New Jersey strains reacted alike while the Palestine strain belonged to another serological group.

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Effect of Antihistamines on Loss of Adhesiveness of Corneal Epithelium After Injection of Histamine.

HEINZ HERRMANN. (Introduced by J. S. Nicholas.)

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In a previous paper¹ we described the effect of histamine on the adhesiveness of the corneal epithelium of excised bovine corneas. In amounts from 0.5 to 1.0 μ g per cornea (600-800 mg wet weight) histamine caused a marked decrease in epithelial adhesiveness. In contrast to other agents which decrease adhesiveness by disruption at the stroma epithelium boundary, histamine seems to effect the detachment of the upper layers of the epithelium from the basal one.

In view of the relatively high amounts of histamine required for this effect (about 10-100 times more than for relaxation of cats intestine) and of the gradual culmination of epithelial loosening it seemed desirable to compare the action on the corneal epithelium of histamine alone and of mixtures of histamine with certain antihistamines. The same technic was used as described previously.² Between 0.04 to 10 μ g of the antihistamines were injected together with a constant amount of 2 μ g of histamine in a total volume of 0.2 ml per cornea. At this concentration the antihistamines were found to have no effect on the adhesiveness of the corneal epi-

thelium. On injection of 50 μ g per cornea or more, toxicity with loosening of the epithelium was observed for all antihistamines tested so far. The indicated amount of histamine was sufficient to cause loosening in all samples. The corneas were incubated for 12-15 hours at 28-30°C. The adhesiveness was tested with a simple scraper which allows a semi-quantitative determination of the adhesiveness, the amount of removable epithelium being a function of the adhesiveness and of the weight on the scraping blade.² The procedure was simplified in the present series of experiments by keeping the pressure on the scraper constant (60 g) and recording the removal of more than 2/3 and of less than 1/3 of the epithelium of the corneal test strip. After injection of histamine alone there was always under these conditions a removal of more than 2/3 of the epithelium. If antihistamines were injected simultaneously the loss of adhesiveness was reduced to less than 1/3 depending upon the concentration of the respective compound.

In Table I our results are summarized designating protection with removal of less

¹Herrmann, H., *Bull. Johns Hopkins Hosp.*, 1948, 82, 208.

²Herrmann, H., and Hickman, F. H., *Bull. Johns Hopkins Hosp.*, 1948, 82, 182.

TABLE I.
Antagonism to Loosening of Corneal Epithelium After Injection of Histamine in its Dependence Upon Concentration of Antihistamines.

Amt of antihistamine per cornea in μ g	Pyribenzamine	PTDA (01013)	Antistine
10.0	++		+++
5.0	+++++		++--
2.5	++++-	+++++	
1.0	+++++	+++++	
0.6	+++++	+++++	
0.4	+++++	+++++	
0.3	+++++		
0.2	+++++	+++++	
0.1	+++++	++--	
0.08	+++++		
0.04	++--		

+ Indicating removal of less than 1/3 of epithelium (protection).

-- Indicating removal of more than 2/3 of epithelium (non-protection).

Each sign stands for one cornea tested. Amount of histamine injected per cornea: 2 μ g.

than 1/3 with + and absence of protection with removal of more than 2/3 of the epithelium with --. It can be seen that pyribenzamine and N-(2-pyridyl)-N-(2-thenyl)-N', N'-dimethylethylenediamine hydrochloride* provide protection in concentrations of less than 1/10 of that of histamine while the protection by Antistine requires a concentration of 2-5 times that of histamine.

* This compound is commercially designated as 01013 and is listed in the table by the initials PTDA.

Summary. The loosening of the upper layers of the epithelium of the excised bovine cornea by histamine is prevented by simultaneous injection of certain antihistamines. Three compounds found to be effective are Pyribenzamine, 01013, and Antistine.

We are indebted for the supply of the antihistamines to Dr. B. N. Craver of the Ciba Pharmaceutical Products, Inc. (Pyribenzamine, Antistine), and to Dr. K. K. Chen of Eli Lilly Co. (01013).

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Serum Polysaccharide Level in the Normal State.*

M. R. SHETLAR, JANEAL VILLET FOSTER, KEITH H. KELLY AND MARK R. EVERETT.

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Several investigations concerning the polysaccharides of serum in both normal and pathological conditions have recently been published. Seibert and Atno,¹ and Seibert, Seibert, Atno, and Campbell² have presented

results of quantitative studies using the carbazole method. Hinsberg and Merten³ published results using an acid hydrolysis procedure while Lustig⁴ *et al.* have reported several studies using an orcinol method. Nil-

* Aided by a grant from the Oklahoma Division of the American Cancer Society and from the John Archer Hatchett Memorial Fund.

¹ Seibert, F. B., and Atno, A. J., *J. Biol. Chem.*, 1946, 163, 511.

² Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W., *J. Clin. Invest.*, 1947, 26, 90.

³ Hinsberg, K., and Merten, R., *Z. Klin. Med.*, 1938, 135, 76; *Brit. Chem. Physiol. Absts.*, 1939A, III, 357.

son⁵ reported elevation of the glucosamine level of serum in pneumonia. His values for average normals were: for adults 77 mg % and for fetal serum 32 mg %; pneumonia serum averaged 183 mg %. West, Clarke, Kennedy⁶ reported elevations in serum glucosamine in infections, disseminated malignant disease, and sterile infarcts.

As this laboratory is engaged in a study of the animal polysaccharides in relationship to malignancy, using a tryptophane method⁷ for the determination of non-glucosamine serum polysaccharides it was necessary to investigate the normal polysaccharide pattern as well as that of as many pathological conditions as possible. It is the purpose of this paper to present the results of a complete study of normal subjects. The results of our study of pathological conditions will be published at a later date.

In order to give a more complete picture, non-glucosamine polysaccharide, glucosamine, tyrosine, and total protein determinations were made on ethanolic precipitates from the same serum.

Experimental. Chemical methods. Non-glucosamine polysaccharide was determined as described in a previous publication.⁷ Total protein was determined, after precipitation of the serum protein with ethanol, by a micro kjeldahl procedure.

Preliminary work with the West, Clarke and Kennedy⁶ method for determining glucosamine indicated that a considerable coloration could be developed with serum hydrolysates without the acetylacetone coupling reaction, consequently the method was modified as follows to give a blank for each hydroly-sate.

Reagents. 1. Absolute Ethanol. 2. 8 N

⁴ Lustig, B., and Langer, A., *Biochem. Z.*, 1931, 242, 321; Lustig, B., and Nassau, E., *Am. Rev. Tuberculosis*, 1941, 43, 817; Novak, J., and Lustig, B., *J. Mount Sinai Hosp.*, 1947, 14, 534.

⁵ Nilsson, I., *Biocem. Z.*, 1937, 291, 254.

⁶ West, R., Clarke, D. H., and Kennedy, E. M., *J. Clin. Invest.*, 1938, 17, 173.

⁷ Shetlar, M. R., Foster, J. V., and Everett, M. R., *Proc. Soc. Exp. Biol. and Med.*, 1948, 67, 125.

Hydrochloric Acid. 3. 2.5 N Sodium Hydroxide. 4. 2.5 N Hydrochloric Acid. 5. Acetylacetone Solution: 0.2 ml of acetylacetone dissolved in 10 ml of 0.5 M Sodium Carbonate (53 g Na_2CO_3 per liter of solution). This solution must be freshly prepared before using. 6. Ehrlich's reagent: Dissolve 5 g of p-dimethylaminobenzaldehyde in 190 ml of absolute ethanol and then add 190 ml of concentrated hydrochloric acid.

Procedure. Add 1 ml of serum-drop by drop to 18 ml of absolute ethanol contained in a 15 x 150 mm pyrex test tube. Allow to stand 5 minutes and centrifuge. Pour off the supernatant liquid and invert the tube to drain the excess alcohol from the precipitate. Add 2 ml of water to the precipitate and stir with a glass rod. Add 2 ml of 8 N HCl and hydrolyse the mixture in a boiling water bath under reflux for 4-5 hours.

Filter through Whatman No. 1 filter paper into 10 ml volumetric flasks. Make up to volume and mix. Pipet 1 to 2 ml of the solution into three 15 ml calibrated centrifuge tubes, add a drop of phenolphthalein indicator to each and carefully neutralize with 2.5 N sodium hydroxide. Make slightly acid by adding a drop of 2.5 N hydrochloric acid. Add water to bring total volume to 3.5 ml. Add 2 ml of the acetylacetone solution to 2 of the tubes and 2 ml of 0.5 M Na_2CO_3 to the third, which serves as a blank. Stopper lightly and place in a boiling water bath for 20 minutes.

Remove from bath and cool below 37.5°C. Make volume to 10 ml with absolute ethanol. Add exactly 2 ml of Ehrlich's solution and mix. Stopper lightly and incubate in 37.5°C oven for 45 minutes. Determine optical density in Coleman 11 Spectrophotometer at a wave length of 540 m μ . Read each sample against its respective blank. A standard containing 100 μg of glucosamine-HCl (83.4 μg glucosamine) is subjected to the same procedure with each set of samples.

Calculations

$$\text{Kx} \times 83.4 \times 10 = \frac{\mu\text{g of glucosamine per ml of serum}}{\text{Ks}}$$

$\text{Ks} \times \text{V}$

Kx = optical density of sample

Ks = optical density of standard

TABLE I.
Summary of the Non-glucosamine Polysaccharide, Glucosamine, Total Protein and Tyrosine Content of Normal Serum.

Group	No.	Polysaccharide, mg %	Polysaccharide, as % of protein	Glucosamine, mg %	Total protein, %
Fetal	15	80 (62-103)	1.41 (1.05-1.77)	48 (42-55)	5.68 (4.26-6.76)
Children (3-8 yrs)	8	105 (94-118)	1.60 (1.47-1.82)	63 (52-69)	6.59 (5.40-6.48)
Young adults:					
Males (21-32 yrs)	18	110 (93-126)	1.58 (1.26-2.02)	66 (62-78)	6.98 (6.00-7.48)
Females (22-49 yrs)	10	111 (100-125)	1.58 (1.42-1.81)	68 (61-78)	7.01 (6.26-7.35)
Aged (61-85 yrs)	15	129 (104-138)	1.79 (1.62-2.06)	81 (70-89)	7.20 (7.09-7.68)

(Figures in parenthesis indicate the high and low figures in each group.)

V = volume of aliquot of hydrolysate

Selection of Subjects. A group of young adults was selected at random from volunteer medical students who had no recent history of any pathological condition. The children's group was obtained from a local orphanage. The aged group comprised selected subjects from the outpatient clinic who had only psychosomatic complaints or fully matured cataracts, or patients selected from a State Mental Hospital. The latter patients were selected with the aid of the institutional staff who were familiar with the condition of each patient through observation over a considerable period of time. These patients were in excellent physical condition aside from mental deterioration. The fetal blood samples were drawn by needle from the umbilical vein of the cord on the placental side immediately after clamping and cutting the cord.

Results. As it is impossible to include the actual analyses of all cases, a summary of normals is shown in Table I. The original data were treated to statistical analysis, in which the averages of the different groups were compared by the following formula from Rider.⁸

$$t = \frac{X_1 - X_2}{\left(\frac{N_1 + N_2}{N + N - 2} \right)^{1/2} \left(\frac{N_1 S_1^2 + N_2 S_2^2}{N_1 N_2} \right)^{1/2}}$$

X_1 and X_2 are the averages of the respective groups, N_1 and N_2 are the number of individuals in the respective groups, and S_1^2 and S_2^2 are the variances of the respective groups. This comparison is summarized in Table II.

From the data it appears that both the poly-

saccharide and glucosamine associated with serum protein tends to increase with age. Levels of fetal individuals are significantly lower than those of young adults, while levels of aged persons are significantly elevated. The levels for children were found to be lower than those for young adults, but the differences were not striking. The difference is not entirely proportional to levels of the serum protein in the various groups, although fetal serums were significantly lower in protein content. When the polysaccharide content for each sample is divided by the corresponding total protein, and the data treated to statistical comparison, the "t" value for the fetal group as compared to the young adult group is 2.669, a figure which is significant at the 2% level.

Since the interrelationships between the different components are important, total protein, non-glucosamine polysaccharide, and glucosamine levels of sera from the young adults were tested for correlation. The multiple correlation coefficient for these three factors was found to be 0.601, a value which is significant at the 1% level ("t" value = 3.76). The partial correlation between protein and polysaccharide independent of glucosamine, was found to be 0.576, and the partial correlation between polysaccharide and glucosamine, independent of total protein, was found to be 0.658. Both of these figures are significant at the 1% level. The correlation between glucosamine and total protein, independent of non-glucosamine polysaccharide was found to be 0.251, a figure which failed to be significant. The reason for this lack of correlation is not immediately apparent.

If all the polysaccharides of serum are composed of equimolecular proportions of man-

⁸ Rider, P. R., Modern Statistical Methods, John Wiley & Sons, New York.

son⁵ reported elevation of the glucosamine level of serum in pneumonia. His values for average normals were: for adults 77 mg % and for fetal serum 32 mg %; pneumonia serum averaged 183 mg %. West, Clarke, Kennedy⁶ reported elevations in serum glucosamine in infections, disseminated malignant disease, and sterile infarcts.

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Remove from bath and cool below 37.5°C. Make volume to 10 ml with absolute ethanol. Add exactly 2 ml of Ehrlich's solution and mix. Stopper lightly and incubate in 37.5°C oven for 45 minutes. Determine optical density in Coleman 11 Spectrophotometer at a wave length of 540 mμ. Read each sample against its respective blank. A standard containing 100 μg of glucosamine-HCl (83.4 μg glucosamine) is subjected to the same procedure with each set of samples.

Calculations

$$Kx \times 83.4 \times 10$$

$$= \frac{Ks \times V}{\text{serum}} = \mu\text{g of glucosamine per ml of serum}$$

$$Kx = \text{optical density of sample}$$

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⁴ Lustig, B., and Langer, A., *Biochem. Z.*, 1931, 242, 321; Lustig, B., and Nassau, E., *Am. Rev. Tuberculosis*, 1941, 43, 817; Novak, J., and Lustig, B., *J. Mount Sinai Hosp.*, 1947, 14, 534.

⁵ Nilsson, I., *Biocem. Z.*, 1937, 291, 254.

⁶ West, R., Clarke, D. H., and Kennedy, E. M., *J. Clin. Invest.*, 1938, 17, 173.

⁷ Shetlar, M. R., Foster, J. V., and Everett, M. R., *Proc. Soc. Exp. Biol. and Med.*, 1948, 67, 125.

normal persons including fetal, children, young adult, and aged representatives.

The serum polysaccharide (both non-glucosamine and glucosamine) was lowest in the fetal group and highest in the aged representatives, the children and young adult groups being intermediate, thus showing a tendency to increase with age.

Concentrations of serum polysaccharide (both non-glucosamine and glucosamine) for young adults were significantly higher than those of fetal serums, and significantly lower than for the aged. The total protein for young

adults was significantly higher than in fetal sera. Significant positive correlations were found between non-glucosamine polysaccharide and total protein and also between non-glucosamine polysaccharide and glucosamine.

In the same individual, non-glucosamine serum polysaccharide and glucosamine were subject to about the same variation as serum protein over a period of sixteen months. Small variations in diet appeared to have little effect. No appreciable changes occurred in serum polysaccharides during the menstrual cycle.

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Failure of Trypan Red to Protect Against Certain Neurotropic Viruses ("M.M." and Russian Spring-Summer Encephalitis).*

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Investigations by Aird¹ on brilliant vital red and by Aird and Strait² on trypan red have shown these supra-vital dyes to affect the distribution of intravenously injected cocaine within the central nervous system. This was presumably due to an alteration of the permeability of the blood-brain barrier since the amount of cocaine reaching the cerebral cortex of cats receiving the dyes was decreased by 31% as compared with the controls not receiving the dyes. As a result of these studies and others, the question was raised as to the possible protective effect of these substances against infection of the central nervous system by neurotropic viruses. Due to the interruption of our studies by the late war we were unable to engage in experimental work along these lines.

Wood and Rusoff³ claimed to demonstrate the protective effect of trypan red on the "M. M." virus.⁴ Unfortunately, Wood and Rusoff inoculated the dye repeatedly by the intraperitoneal route and subsequently inoculated the virus by the same route. It has been the general experience of bacteriologists and immunologists that a local, non-specific protection against most infectious agents is set up in the peritoneal cavity by many types of inert substances when given previously by the same route.

Further studies on the possible protective effect of the supra-vital dyes against virus infections of the central nervous system, therefore, appeared indicated. Considering the mode of action of the dyes, it seemed wise to select viruses capable of infecting the central nervous system when inoculated by a peripheral route, as opposed to viruses requiring direct inoculation into the central

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc., and the Christine Breon Fund.

¹ Aird, R. B., *Arch. Neurol. and Psychiat.*, 1939, 42, 700.

² Aird, R. B., and Strait, L., *Arch. Neurol. and Psychiat.*, 1944, 51, 54.

³ Wood, H. G., and Rusoff, I. I., *J. Exp. Med.*, 1945, 82, 297.

⁴ Jungeblut, C. W., and Dalldorf, G., *Am. J. Pub. Health*, 1943, 33, 169.

TABLE II.
 Statistical Comparison of Results on Normal Subjects.

Constituent	Groups compared	Difference %	t	D.F.
Total protein	Male v. female	0.03	1.280	26
Polysaccharide	" " "	1.13 mg	0.066	26
Glucosamine	" " "	1.56 "	0.648	26
Total protein	Young adults v. fetal	1.31	7.941*	41
Polysaccharide	" " " "	30.37 "	9.230*	41
Glucosamine	" " " "	19.13 "	12.003*	41
Total protein	Young adults v. children	0.40	2.185†	34
Polysaccharide	" " " "	5.45 "	1.445	34
Glucosamine	" " " "	3.75 "	1.797	34
Total protein	Young adults v. aged	— 0.22	1.707	41
Polysaccharide	" " " "	—18.22 "	6.107*	41
Glucosamine	" " " "	—13.53 "	7.517*	41

* Significant at the 1% level.

† Significant at the 5% level.

nose, galactose, and glucosamine, the glucosamine content divided by the non-glucosamine polysaccharide should be 0.498. The actual average figures were found to be as follows: Fetal 0.593, children 0.598, young adults 0.609, aged 0.626, weighted average 0.607.

The probability that the difference between the actual figure (0.607) and the theoretical value (0.498) could have arisen by chance is only 2.33 in 100, consequently it appears that all serum polysaccharides are not polymers of galactose-mannose-glucosamine in equimolecular proportions. There also appears to be a tendency for the ratio to increase with age, however this increase was not significant on the basis of the data obtained in this study.

Variation from time to time. In order to check the variability of serum polysaccharide in an individual, from time to time, samples were secured periodically over a period of 16 months. During this period the non-glucosamine polysaccharide varied between 110 and 122 mg %, the glucosamine between 70 and 78 mg % and the protein between 7.20 and 7.95%. This variation in polysaccharide is somewhat smaller than that recorded by Seibert and Atno,¹ and suggests that the polysaccharide level in healthy individuals is subject to about the same variation as the serum protein.

Effect of Diet. No significant difference was found in the polysaccharide levels determined on the same person fasting, 1 hour

after a large meal, or 4 hours after the same meal (non-glucosamine polysaccharide levels of 115, 122 and 120 mg % respectively). Oral administration of 25 g mannose, 100 g galactose, or 50 g glucosamine caused no significant change in the polysaccharide level after 3 hours, 24 hours, or 72 hours. The polysaccharide levels were not appreciably affected by fasting (for 31 and 55 hours), by a high protein diet, by a low protein diet, or by high water intake (for 33 hours).

Stability of serum polysaccharide during storage. Data obtained from serum kept in the refrigerator for various lengths of time are recorded in the following table:

Days stored	Polysaccharide	T. protein	Glucosamine
0	120	7.20	75
1	117	—	73
2	115	—	73
3	114	—	76
7	115	7.32	74

Effect of menstrual cycle. Polysaccharide, glucosamine, and total protein determinations were made on the serum of 2 normal females at various times during the menstrual cycle (on the 2nd, 4th, 14th, 17th, and 22nd days of the cycle). Both subjects had normal 28 day cycles. The polysaccharide level varied between 111 and 118 mg % in one subject and 108 and 124 mg % in the other with no apparent correlation between the menstrual cycle and the polysaccharide level.

Summary and conclusions. Studies of serum polysaccharides were made on a total of 66

terpret the apparent protective effect obtained by Wood and Rusoff³ as being due to a well recognized and non-specific peritoneal protection afforded by certain inert substances when injected previous to the inoculation of infectious agents by the same route. Our results suggest that these two neurotropic viruses do not enter the central nervous sys-

tem directly by way of the vascular channels as is the case with such an agent as cocaine, the passage of which into the central nervous system tissue may be altered by trypan red.

We wish to acknowledge the technical assistance of Doctor David Zealear and Miss Sylvia Bowditch in one phase of this study.

16773

Unidentified Growth Factor(s) Needed for Optimum Growth of Newborn Pigs.

A. L. NEUMANN, J. L. KRIDER AND B. CONNOR JOHNSON

From the Department of Animal Science and the Division of Animal Nutrition, University of Illinois, Urbana.

An unidentified growth factor(s) (the animal-protein factor, factor X,¹ vitamin B₁₂,² vitamin B₁₃,³ etc.) has been reported to be present in certain concentrates such as anti-pernicious anemia liver extract. It has been reported that this factor(s) will improve the growth rate of the rat,⁴ the dog,⁵ the fox,⁶ and the chick.⁷

Since it has been shown^{8,9} that newborn pigs can be raised successfully to weaning age on a synthetic ration made up to simulate milk, this appeared to be a suitable technic for determining whether the pig requires such an identified growth factor(s). Carey *et al.*¹

have reported that casein supplies significant amounts of factor X to the rat, and for this reason the "vitamin-free" casein used in previous studies^{8,9} was replaced in the first experiment by an isolated soybean protein (alpha-protein).^{*} Methionine was added to the diet, since this alpha-protein is deficient in sulfur amino acids.¹⁰ The composition of the rations fed is given in Table I.

Experimental. Experiment I. Seven 2-day-old Duroc Jersey-Chester White cross-bred pigs from the same litter were divided into 3 groups on the basis of weight and age. The pigs were housed in individual raised wire cages in a heated building and were fed the following diets *ad libitum*. Group 1 received the casein "synthetic milk" ration⁹ (Diet A, Table I); Group 2 received the alpha-protein "synthetic milk" (Diet B); and Group 3 received Diet B plus Reticulogen,[†] a 20-unit/ml anti-pernicious anemia liver extract. Individual feed consumption records were

¹ Cary, C. A., Hartman, A. M., Dryden, L. P., and Likely, G. D., *Fed. Proc.*, 1946, **5**, 128.

² Riekes, E. L., Brink, N. G., Koniusky, F. R., Wood, T. R., and Folkers, K. F., *Science*, 1948, **107**, 396.

³ Novak, A. F., and Hauge, S. M., *J. Biol. Chem.*, 1948, **174**, 647.

⁴ Jaffe, W. G., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **169**, 287.

⁵ Ruegamer, W. R., Torbet, N., and Elvehjem, C. A., *Fed. Proc.*, 1947, **6**, 187.

⁶ Schaefer, A. E., Whitehair, C. K., and Elvehjem, C. A., *Fed. Proc.*, 1947, **6**, 420.

⁷ Novak, A. F., Hauge, S. M., and Carriek, C. W., *Poultry Sci.*, 1947, **26**, 604.

⁸ Johnson, B. C., James, M. F., and Krider, J. L., *J. Animal Sci.*, 1947, **6**, 486.

⁹ *Ibid.*, *J. Animal Sci.*, 1948, **7**, 486.

¹⁰ Grau, C. R., and Almquist, H. J., *J. Nutrition*, 1943, **26**, 631.

^{*} The alpha-protein was generously supplied by the Glidden Co., Chicago, Ill., through the courtesy of Dr. J. L. Gabby.

[†] The reticulogen (20-unit anti-pernicious anemia liver extract) was generously supplied by Eli Lilly and Co., Indianapolis, Ind., through the courtesy of Dr. E. C. Kleiderer.

TABLE I.
Effect of Trypan Red on Russian Spring-Summer Encephalitis Virus in Mice.

Date of experiment	No. of mice	LD ₅₀ controls	LD ₅₀ trypan red	LD ₅₀ Difference
8/27/46	60	5.75	5.75	0.00
1/20/47	78	5.76	6.44	+0.68

TABLE II.
Effect of Trypan Red on "M.M." Virus in Mice.

Date of experiment	No. of mice	LD ₅₀ controls	LD ₅₀ trypan red	LD ₅₀ Difference
5/23/47	47	5.63	5.52	-0.11
23	47	6.42	6.38	-0.04
7/16	80	6.08	5.68	-0.40
10/25	189	6.48	6.33	-0.15

nervous system. Our experiments were, therefore, planned to employ the same virus used by Wood and Rusoff, and in addition the Russian spring-summer encephalitis virus (Sophy strain-eastern type).[†] Both of the viruses infect readily by the intraperitoneal and other peripheral routes. The route or mode of invasion of the central nervous system, however, is as yet unknown for both viruses. It is entirely possible that they are not carried by the blood stream (as is cocaine) but may invade through peripheral nerves.

Mice were inoculated repeatedly as indicated below, with 1% trypan red in saline by the subcutaneous route over a period of several days. This resulted in their skin assuming a bright reddish tint. Controls were given saline in the same dosage. Following this preparation all mice were inoculated with one or the other of the viruses by the *intraperitoneal* route. Serial dilutions of the virus were prepared from frozen ampoules and four 10-fold dilutions were selected to cover the range of virus from that which would kill all mice to that which would not kill any. The LD-50 was calculated by the method of Reed and Muench.⁵

Russian Spring-summer virus. The mice were prepared by 7 subcutaneous inoculations of 0.1 to .05 cc of 1% trypan red or with

saline over a period of 4 weeks. Five days after the last injection the virus was inoculated intraperitoneally. The results of 2 consecutive experiments employing 138 mice are shown in Table I. It will be noted that, if any effect was produced, the mice inoculated with the dye were the more susceptible. In our opinion, however, the difference shown is not significant.

"M. M." virus. In these experiments less dye was given. The dosage given by Wood and Rusoff was followed exactly: 0.1 cc of a 1% solution being injected on 3 successive days. The virus was given one day after the last dye injection. As in all our tests the dye was given subcutaneously and the virus intraperitoneally. The results of 4 successive experiments employing 363 mice, are shown in Table II. It may be observed that the differences between the controls and those inoculated with dye ranged from 0.11 to 0.40 log and never attained a significant difference. However, in each experiment the small differences which did appear always occurred in the same direction; a lower LD-50 in those receiving the dye. It was felt that this effect, however, could well be due to chance alone.

Conclusions. Trypan red failed to produce a significant protection against Russian Spring-summer encephalitis virus and "M.M." virus when the dye was injected subcutaneously and the virus was inoculated by the intraperitoneal route. These experiments fail to confirm the results of others who injected both dye and virus intraperitoneally. We in-

[†] Obtained through the courtesy of the Army Medical Department Virus and Rickettsia Laboratories.

⁵ Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, 27, 493.

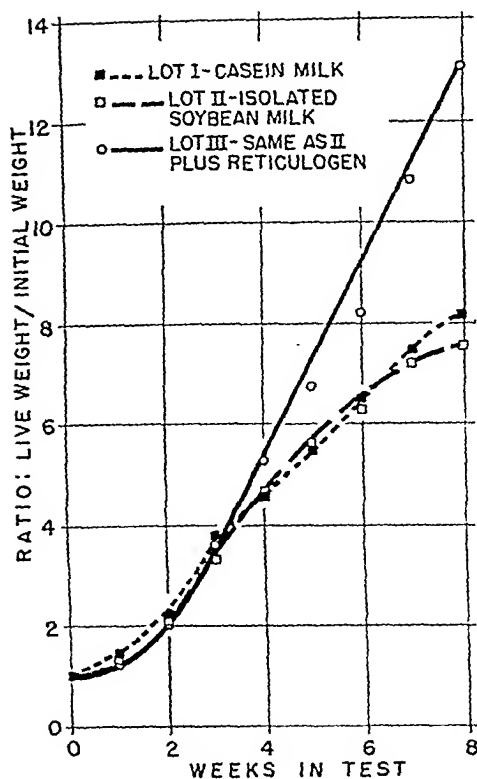


FIG. 1.

Average gains expressed as ratio of weight to initial weight.

improvement in thrift, size, condition and amount of haircoat, and soundness of feet and legs when Reticulogen was given as a source of the unidentified growth factor(s).

Normal hemoglobin levels and normal red and white cell counts were found in all pigs at 8 weeks.[§]

At the close of the experiment all pigs were placed together and given access to green pasture and a good farm ration for weanling pigs. After 3 weeks there had been practically no gain in the pigs from Groups 1 and 2, while those from Group 3 continued to gain normally. Only after 5 weeks on a good farm ration did the pigs on the unsupplemented rations overcome their growth factor(s) deficiency.

Experiment 2. In order to confirm the results of Experiment 1 in which only a small number of animals were used, a second experiment was carried out in which 21 two-day-old Duroc-Jersey pigs were used. In this experiment vitamin-free casein was used as the protein source in the ration, since the results of Experiment 1 had indicated that this vitamin-free casein was almost as deficient in the unknown factors present in Reticulogen as was alpha-protein.

The basal ration (Diet A, Table I) was fed to a group of 9 baby pigs. Another group of 12 baby pigs received the same ration plus 0.25 ml of Reticulogen per pig daily. The pigs were housed and fed as in Experiment 1.

Again, after the 8-week experimental period, the animals in the group receiving Reticulogen had gained significantly more than those on the basal ration ($P = <0.001$). These results are summarized in Table III.

¹¹ Fisher, R. A., Statistical Methods for Research Workers, 9th Ed., Oliver and Boyd, Ltd., London, 1944.

[§] We are indebted to Dr. Marian F. James for these determinations.

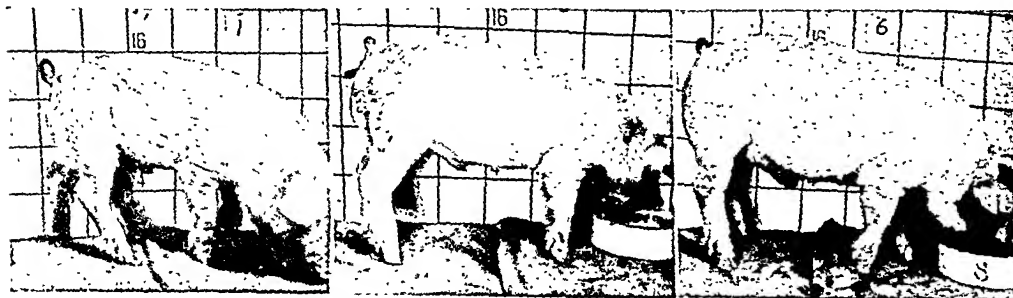


FIG. 2.

Representative pigs from the 3 lots. Left, Pig No. 1 from the casein lot. Middle, Pig No. 3, from the alpha-protein lot. Right, Pig No. 6, from the alpha-protein + Reticulogen lot.

TABLE I.
Composition of "Synthetic Milk" Rations.

	Diet A Casein diet. (Group 1)	Diet B Alpha protein diet (Group 2 and 3)
Casein (Labeo, vitamin-free)	30	
Alpha protein (isolated soybean protein)	—	29.7
Methionine*	—	0.3
Glucose (cerelose)	37.4	37.4
Mineral salts†	6.0	6.0
Lard	26.6	26.6
Reticulogen	—	0.33 ml/pig/day Group 3, Expt. I

Made up and homogenized into a milk containing 13% solids including 4% lard (liquid basis).

The following vitamins‡ were added per 1000 g of milk:

Thiamine	0.65 mg	p-aminobenzoic acid	2.6 mg
Riboflavin	1.30 "	Pteroylglutamic acid	0.052 "
Pyridoxine	1.30 "	Biotin	0.01 "
Calcium pantothenate	7.8 "	α -tocopherol acetate	1.0 "
Inositol	26.0 "	2-methyl-1,4 naphthoquinone	0.26 "
Choline	260.0 "	Vit. A	1000 I.U.
Nicotinic acid	2.67 "	Vit. D	100 "

* The *dl*-methionine was furnished by E. I. DuPont de Nemours and Company, Inc., New Brunswick, N.J.

† See Johnson *et al.*⁹

‡ The thiamine chloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, nicotinic acid, biotin, and α -tocopherol acetate used in this experiment were generously supplied by Hoffman-La Roche, Inc., Nutley, N. J. Pteroylglutamic acid was supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y. Inositol was donated by A. E. Staley Manufacturing Co., Decatur, Ill. Hyflavin (a highly water-soluble riboflavin) was furnished by Endo Products, Inc., New York City.

TABLE II.
Results of Experiment I.

Items compared	Groups and treatments		
	I	II	III
	Casein milk	Isolated soybean protein milk plus DL-methionine	Same as II plus reticulogen
No. of pigs started	2	2	3
Av initial wt, g	1780	1785	1523
" wt at 21 days, g	6940	6125	5600
" final wt, g	14610	13530	19960
" daily gain during test, g	231.5	209.8	326.9
" " " to 21 days, g	245.2	206.6	195.2
" " " 21 to 56 days, g	222.5	211.6	404.1

kept and the pigs were weighed at weekly intervals.

Reticulogen was given to the pigs in Group 3 at the rate of 0.33 ml per pig daily. To reduce intestinal synthesis of unidentified growth factors, sulfathaladine‡ was added to the diet at the rate of 2 g per liter of milk. The fat-soluble vitamins were added to the

milk during homogenization, and the water-soluble vitamins were added to the milk at the time of feeding.

The gains as ratios of attained weight to initial weight for the 3 groups are plotted in Fig. 1, and the data are summarized in Table II. At 8 weeks the pigs in Group 3 (Reticulogen) had gained significantly more than those in Group 2 ($P = 0.007$)¹¹ or those in Groups 1 and 2 ($P < 0.001$).¹¹

The photographs in Fig. 2 illustrate the

‡ Sulfathaladine (phthalylsulfathiazole) was generously supplied by Sharp and Dohme, Philadelphia, Pa., through the courtesy of Dr. S. F. Sheidy.

TABLE I.

Treatment	Total No. animals	No. animals without growth	No. of animals with growth						
			1 wk	2 wk	3 wk	4 wk	5-7 wk	8-12 wk	12-16 wk
Glidden									
Progesterone	26	4	1(1)	11(10)	20(9)	22(2)	22(0)	22(0)	22(0)
Oestradiol	18	9	0	0	0	2(2)	4(2)	9(5)	—
Progesterone*	8	7	0	0	0	0	0	0	1(1)
Control	18	14	0	0	0	0	0	1(1)	4(3)

Incidence and time of apparent subcutaneous growth of tumor 18C57) with different hormone treatment.

The numbers in brackets are the numbers of animals showing first growth in that period while the unbracketed numbers are total tumor takes through that period.

* "Proluton" obtained through the courtesy of the Schering Corp., Bloomfield, N. J., containing 5 mg progesterone/cc.

TABLE II.

Treatment	Total No. animals	No. animals showing growth in 6 wk
Glidden Progesterone 0.05 cc two times per week	31	28
Oestradiol 0.05 cc two times per week	9	5
Progesterone* ("Proluton") 0.1 cc two times per week	4	0

Number of animals treated and showing growth of tumor within 6 weeks with 3 types of hormone treatment.

* "Proluton" obtained through the courtesy of the Schering Corp., Bloomfield, N. J., containing 5 mg progesterone/cc.

Growth of one of the transplanted tumors occurred but rarely and only after a long time in intact or castrated animals with and without estrogen treatment. In castrated animals treated with the crude progesterone, growth of the tumor occurred in some cases within 10 days. Subsequent study has confirmed this finding plus additional information.

Methods. Mice of the C57 strain approximately 2 to 3 months of age were castrated and a small piece of tumor was transplanted subcutaneously in each animal. The tumor was obtained from a first generation subcutaneous transplant. Injections were started on the day of transplantation. To facilitate injection, the crude material was diluted by adding 1 part Ethanol to 4 parts of the compound. The test animals in the first group received 0.05 ml of the diluted "progesterone compound" 3 times per week. Subsequent series received the diluted material 2 times per week. Control animals were set up in 4 groups: Group one received estradiolbenzoate (25 µg in 0.05 cc sesame oil

2 times a week); Group 2 received Ethanol (1 part to sesame oil 4 parts, 0.05 cc two times per week); Group 3 received progesterone 0.05 cc daily, and subsequently 0.1 cc daily; and Group 4 received no injections. Groups 2 and 4 have been combined in the study of results (Tables I and II). All animals were kept on a standard diet and water *ad libitum*, with the same number of animals per cage. Alternate animals were used for each treatment.

Three series of animals so treated, confirmed the preliminary opinion that the crude, progesterone-containing compound, when injected into animals receiving a transplant of the granulosa cell tumor, stimulated growth of the tumor (Tables I and II). Seventy-seven per cent of the "progesterone compound" treated animals had growing tumor within 3 weeks, and 85% within 4 weeks, while in the other groups no palpable tumors were ob-

|| "Proluton" obtained through the courtesy of the Schering Corp., Bloomfield, N. J., containing 5 mg progesterone/cc.

TABLE III.
Weight Gains in Experiment II.

	Group	
	I Basal ration A	II Basal ration A + reticulogen
No. animals	9	12
Av initial wt	1.56 kg	1.60 kg
Av final wt at 8 wks	16.0 "	21.6 "
Av gain in wt for 8 wks	14.4 "	20.0 "

Summary. The addition of Reticulogen (a 20-unit anti-pernicious anemia liver extract) to a synthetic milk diet resulted in an increased growth rate in baby pigs over an 8-week period. The final weights of pigs receiving Reticulogen averaged 19.96 and 21.6 kg as compared with 13.53 and 16.0 kg for those not receiving Reticulogen in two comparisons.

16774

Effect of a Progesterone Compound on Growth of a Transplanted Granulosa Cell Tumor.*

EUGENE E. CLIFTON† AND SHIH-CHENG PAN.‡ (Introduced by S. C. Harvey.)

From the Departments of Anatomy and Surgery, Yale University School of Medicine.

Progesterone has been considered to be inhibitory to, or without effect on, tumor growth. Heimann¹ reported a marked decrease in incidence of mammary carcinoma in the RIII strain of mice treated with progesterone alone and a still lower incidence when this was combined with testosterone. He stated, however, that it did not prevent growth of transplanted tumors in mice. He also found^{1b} that it inhibited growth of adenomatous portions of breast fibroadenomata and decreased the number of takes of auto- and homo-transplants in rats. There was no effect on fibroma, myxoma, or sarcoma or on adenomatous growth in pregnant or castrated rats. In the guinea pig Lipschütz and Vargas² observed decrease in

size, and actual disappearance, of the fibroids caused by oestrogen. Burrows and Hoch-Ligeti,³ however, found no change in incidence of mammary carcinoma in mice injected weekly with 1 mg of progesterone, and Loesser⁴ found slight, if any, improvement in animals or patients with breast carcinoma treated with progesterone.

The effects of larger amounts of progesterone on tumor growth have been studied, using a material containing a high concentration of progesterone.⁵ Preliminary experiments indicated a growth-stimulating effect on mammary carcinoma in mice.⁵ A study of the effect of this material on a transplanted ovarian tumor was then undertaken. Subcutaneous transplantation of a granulosa cell tumor induced by intrasplenic transplants⁶ in mice of the C57 strain had been attempted.

* This investigation was supported in part by grants from the Jane Coffin Childs Memorial Fund for Medical Research, and the United States Public Health Service Grant C343, administered by W. U. Gardner.

† Senior Fellow, American Cancer Society, as recommended by the National Research Council.

‡ Anna Fuller Fund Fellow. Present address: Hsiang-ya Medical College, Changsha, China.

¹ a, Heimann, Jacob, *Cancer Research*, 1945, 5, 426; b, Heimann, Jacob, *Cancer Research*, 1943, 3, 65.

² Lipschütz, A., and Vargas, L., *Lancet*, 1939, 2, 420.

³ Burrows, Harold, and Hoch-Ligeti, Cornelia, *Cancer Research*, 1946, 6, 608.

⁴ Loesser, A. A., *Lancet*, 1941, 2, 698.

⁵ A crude preparation believed to contain 200-250 I.U. Progesterone/cc made available through the courtesy of E. A. Eugstrom, The Glidden Co., Chicago, Ill.

⁶ Clifton, E. E., unpublished data.

⁷ Li, M. H., and Gardner, W. U., *Cancer Research*, 1947, 7, 549.

TABLE I.

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Growth of one of the transplanted tumors occurred but rarely and only after a long time in intact or castrated animals with and without estrogen treatment. In castrated animals treated with the crude progesterone, growth of the tumor occurred in some cases within 10 days. Subsequent study has confirmed this finding plus additional information.

Methods. Mice of the C57 strain approximately 2 to 3 months of age were castrated and a small piece of tumor was transplanted subcutaneously in each animal. The tumor was obtained from a first generation subcutaneous transplant. Injections were started on the day of transplantation. To facilitate injection, the crude material was diluted by adding 1 part Ethanol to 4 parts of the compound. The test animals in the first group received 0.05 ml of the diluted "progesterone compound" 3 times per week. Subsequent series received the diluted material 2 times per week. Control animals were set up in 4 groups: Group one received estradiolbenzoate (25 µg in 0.05 cc sesame oil

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|| "Proluton" obtained through the courtesy of the Schering Corp., Bloomfield, N. J., containing 5 mg progesterone/cc.

served in the 3 week period and only 2 in the 4 week period. Allowing a period of at least 4 months for the control animals to develop tumors, it was found that 50% of the estrogen treated animals, 22% of the animals receiving Ethanol in sesame oil or no injections, and one, or 25% of the "proluton" treated animals showed tumor growth, to compare with the 85% tumor growth within 4 weeks with the test material.

One small series of intact mice with transplanted tumor, divided into "progesterone compound" treated, estrogen treated, "proluton" treated, and control groups of 3 animals each, was studied. These animals were also allowed to breed. Of this group only one, a progesterone compound treated mouse, developed a tumor.

Subsequent groups of animals with tumor transplants of the second and third generations, observed for only 6 weeks, are shown in Table II. These confirm the previous findings with 90% of the "compound" treated, 55% of the estrogen treated, and none of the "proluton" treated animals showing tumor

growth.

Additional facts in evaluating the action of the "progesterone compound" are: (1) 7 of the "progesterone compound" treated animals of the original 26 died of their tumors within the period 1 to 3½ months after transplantation, while none of the other animals of the original 3 series died of their tumors up to 4 months after transplantation. Many animals were sacrificed in all groups but in every instance the animals in the control groups were permitted to live longer because their tumors were smaller; (2) 2 of the "progesterone compound" treated animals developed gross and several had microscopic metastases, while no animals of the other groups showed metastatic spread.

More detailed studies are being carried out. One obvious line of investigation will be the effect of other hormones especially androgens on the growth of this tumor. It is always possible that this growth stimulation is due to some other material present in the "compound", which can be proven only by more detailed chemical work.

16775 P

Effects of Folic Acid on the Anemia Induced by X Irradiation.

S. PHYLLIS STEARNER. (Introduced by A. M. Brues.)

From the Biology Division of the Argonne National Laboratory, Chicago, Ill.

Previous work in this laboratory has shown that the anemia which followed radiations from internal and external sources was frequently macrocytic.^{1,2} The macrocytosis may have been the result of damage directly to the bone marrow or indirectly through damage to the viscera, resulting in failure to form an anti-anemia factor. Folic acid, classified as a vita-

min of the B complex, is reported to be similar in its action to liver extract. It has been shown to be effective therapeutically in certain of the macrocytic anemias in man.^{3,4} It was without effect, however, upon the macrocytic anemia that resulted from administration of Sr⁹⁰.^{5,6} Total-body X irradiation, unlike radioactive strontium, causes damage to the viscera as well as bone marrow. It is

¹ Stearner, S. P., Simmons, E. L., and Jacobson, L. O., to be published, 1948.

² Simmons, E. L., and Jacobson, L. O., Radio-toxicity of injected Sr⁹⁰ for rats, mice and rabbits. Part IV. The hematological effects of enterally and parenterally administered Sr⁹⁰ in mammals. National Nuclear Energy Series, Vol. 22b.

³ Spies, T. D., Vilter, R. W., Koeh, M. B., and Caldwell, M. H., *Southern Med. J.*, 1945, **38**, 707.

⁴ Moore, C. V., Vilter, R. W., Manneek, V., and Spies, T. D., *J. Lab. and Clin. Med.*, 1944, **29**, 1226.

⁵ Jacobson, L. O., Stearner, S. P., and Simmons, E. L., to be published.

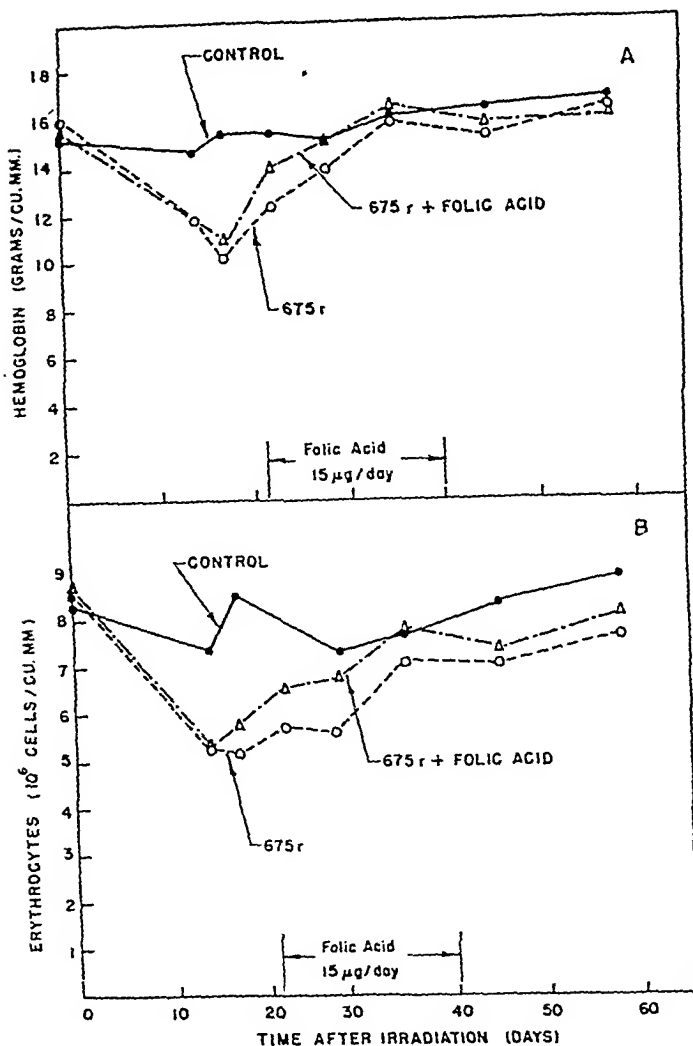


FIG. 1.

Effect of 675 r X irradiation and administration of folic acid on amount of hemoglobin (A) and number of erythrocytes (B) in the peripheral blood.

of interest, therefore, to determine the possible therapeutic effect of folic acid on the macrocytic anemia resulting from total-body X irradiation.

Male white rats were irradiated with 675 r (the approximate median lethal dose). Conditions of irradiation were: 200 Kv, 15 ma, 0.5 mm Cu and 1.0 mm Al filters, target field distance 72.5 cm, and exposure rate about

16 r per minute. One half of the irradiated animals were given intraperitoneal injections of folic acid daily, 5 days per week. The animals were divided into 3 groups and treated as follows:

Group	No.	Treatment
1	10 (5 blood animals)	Controls
2	10 (5 blood animals)	675 r
3	10 (5 blood animals)	675 r, and receiving 15 µg folic acid per day for the period 22-40 days after irradiation

⁶ Jacobson, L. O., Stearner, P., and Simmons, E. L., *J. Lab. and Clin. Med.*, 1947, **32**, 1425, Abst. 37.

EFFECTS OF FOLIC ACID

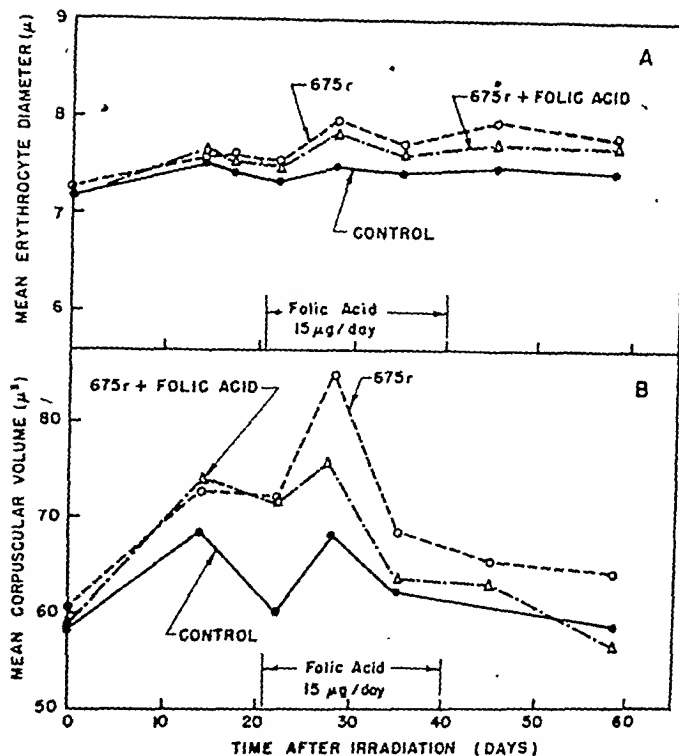


FIG. 2.
Effect of 675 r X irradiation and administration of folic acid on mean erythrocyte diameter (A) and mean corpuscular volume (B).

About 2 weeks after exposure the mean hemoglobin and erythrocyte values in the peripheral blood fell to about 70% of that of the control (Fig. 1). At most sampling intervals the irradiated group that received folic acid showed slightly higher values than the untreated irradiated group. The mean erythrocyte diameter was increased in the irradiated groups at 3 weeks after exposure and persisted for the duration of the observation. Fig. 2 shows the macrocytosis was approximately equal in the 2 irradiated groups and corresponded to the increase in mean corpuscular volume. However, the increase in mean erythrocyte volume was slightly greater in the untreated than in the folic acid treated irradiated group. The greatest increase in per cent of reticulocytes occurred before the maximum mean corpuscular volume was reached (Fig. 3) and therefore could not have caused the increase in mean erythrocyte diameter and

volume. The administration of folic acid had no apparent effect on the reticulocyte response following irradiation.

Analysis of variance⁷ between Group 2 and Group 3, for all sampling periods from 22 to 58 days (inclusive) after irradiation, indicated a significant difference ($P \geq 0.01$) in only the mean erythrocyte count and mean corpuscular volume. (The variance introduced as a result of separate group caging does not affect the statistical results and, for simplicity of presentation, was ignored). The importance of this difference is minimized because the related factors (hemoglobin and mean erythrocyte diameter) did not show a significant difference. It appears, therefore, that there is little or no effect of folic acid upon the erythropoietic system following exposure to X radiation. The damage to the

⁷ Tyler, S., personal communication.

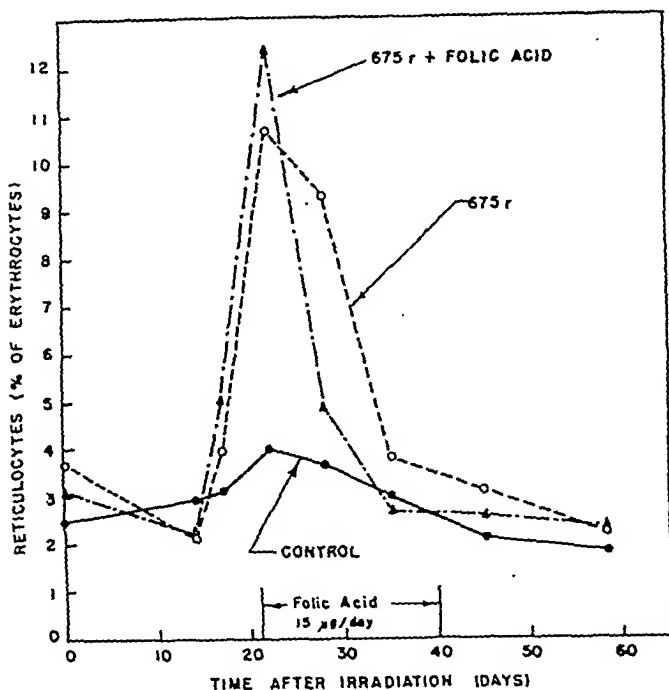


Fig. 3.
Effect of 675 r X irradiation and administration of folic acid on % of reticulocytes in the peripheral blood.

bone marrow is probably caused principally by direct injury. Any indirect damage resulting from damage to the viscera, which

produces the antianemia principle, must be a relatively minor source of injury.

16776

Genetic Changes in Gastric Lesion and Fibrosarcoma Susceptibilities.*

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Evidence has been published that the gene for brown hair has mutated to dominant black several times in the descendants of mice which had been injected with methylcholanthrene for several generations. Concomitant

with the production of these dominant hair color mutations there were also obtained (1) a considerable increase in susceptibility to induced fibrosarcomas, (2) an increased litter size, and (3) increased vitality. All 3 of these characteristics appear to be associated with the black mutant character, never with their brown litter mates. The evidence points toward the production of a widespread germinal change brought about by the effect of methylcholanthrene upon the germ plasm,

* This experiment has been made possible by grants from The Anna Fuller Fund, The Jane Coffin Childs Memorial Fund for Medical Research, and The Committee on Growth of the National Research Council acting for The American Cancer Society.

but a point mutation at a single locus has not as yet been completely excluded.¹

It has also been demonstrated that the gene for susceptibility to induced fibrosarcomas in mice shows linkage relationship with the gene for black hair pigmentation. The essential data of crossing over between these two genes on the brown tagged chromosome have already been obtained.²

A gene for susceptibility to a stomach lesion occurring primarily in the gastric mucosa of NHO mice also shows linkage relationship with the brown gene.³

The present paper reports the data obtained on the incidence of gastric lesions following the subcutaneous injection of methylcholanthrene in the black mutants and their litter mate brown segregates. Of the black mutants 6 descents have been continued for several generations, 3 of these descents were obtained from a brother-sister mating of two of the original black mutants, whereas the other 3 descents were descended from a backcross between one of the black mutants and its own mother of the original brown ancestry. Each of these 3 descents were made up of (a) pure breeding blacks, (b) derived pure breeding brown segregates, and (c) black heterozygous for brown. All mice were weaned at 30 days of age, mated together and injected subcutaneously in the right groin with 1 mg of methylcholanthrene dissolved in 0.1 cc of sesame oil at 60 days of age. They were examined periodically for the appearance of tumors and signs of sickness. All mice were killed when progressively growing tumors were evident or when they showed one or more signs of sickness, such as emaciation, wheezing or a ruffled appearance of the hair.

It is impossible to demonstrate whether these 3 black mutants, which appeared in one litter of mice were derived from one original mutation or from 3 separate ones. If the mutation occurred in one germinal epithelial cell and then the mutant cell gave rise to 3

eggs, it is possible that one mutation was responsible for all 3 mutants. This interpretation is consistent with the embryology of the mouse. There are further considerations that only one mutation was involved. (1) It is highly improbable that any inductive system no matter how specific in action would produce three mutations at the same locus in 3 separate animals without inducing other mutations at other loci. This did not occur. (2) The 3 mutants are the same as far as the somatic manifestation of the mutant gene is concerned. They are each characterized by a peculiar distribution of pigment granules in the hairs which simulates but differs somewhat from the pigment distribution in the well known black mutation, as found in mice of the C₃₇ black stock.³ All 3 black mutants gave comparable Mendelian ratios when crossed with each other or in the F₂ and backcross generations to the recessive condition, brown, following an outcross to mice of the Strong A strain. For these reasons it is considered that the three mutants are genetically and biologically similar if not identical and the data obtained on the descendants of all these can be legitimately classified together.

Of these mice derived from the black mutants and injected with methylcholanthrene, 362 developed lesions involving the stomach, as follows: 198 black to 164 browns. These gastric lesions consisted of several histological types, comparable to the varieties already obtained with methylcholanthrene in the original brown ancestry. There were 3 distinct regions of the stomach of the mouse which gave rise to neoplasia, as follows: (1) a squamous type from the fore-stomach, (2) mixed types from the region of the limiting ridge, and (3) the lesions just anterior to the pylorus involving the mucus secreting neck cells of the gastric mucosa. In the black series the sex differential of gastric lesions was 1.95 males to 1.00 females, whereas in the brown series the sex differential was 2.40 males to 1.00 females. The distributions of gastric lesions for the two color classes of mice are given in Chart 1: brown mice on the short dash line, black mice on the solid line.

¹ Strong, L. C., *Fate J. Biol. and Med.*, 1946, 18, 359.

² Strong, L. C., *Science*, 1946, 103; 554.

³ Strong, L. C., *J. Nat. Cancer Inst.*, 1945, 5, 339.

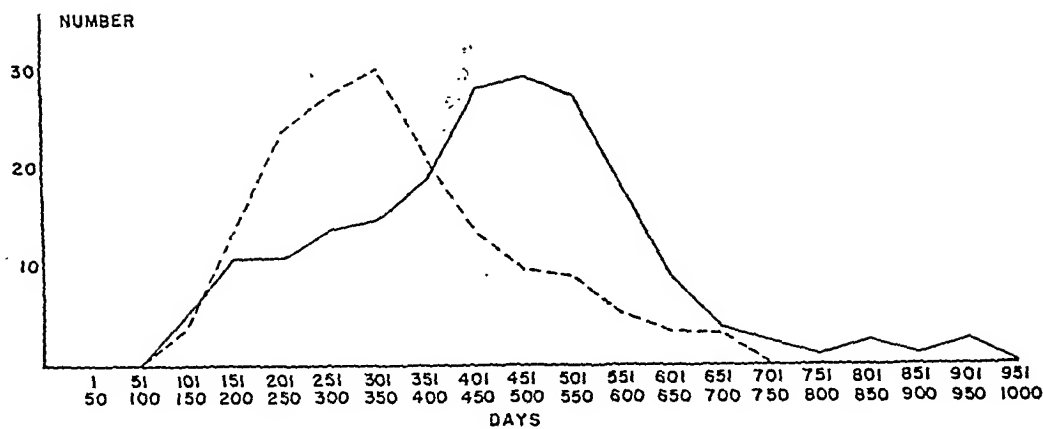


CHART 1.

This chart presents the data on the frequency distribution of induced gastric lesions in mice of the B1 subline; time in days is given on the base line, the number of mice on the vertical line. Black mutants are given on the solid line, brown segregates on the short dash line.

The average latent period for the appearance of the gastric lesions in black mice was 434.4 days, whereas the average latent period for the brown mice was 335.8 days. This is a difference between the two color classes, of 98.6 days. The average latent period of gastric lesions in the original brown descent from which the black mutation had occurred was 318.5 days. Thus it is evident that the brown segregates from the black mutants retain the same latent period for gastric lesions as their brown ancestors (335.8 days-318.5 days = 17.3 days difference) whereas the black mutants have acquired a delayed latent period of 115.9 days beyond the original brown ancestry (434.4 days - 318.5 days = 115.9 days).

The present data on the incidence of tumors induced in black mutants by the subcutaneous injection of methylcholanthrene has therefore,

TABLE I.

	B1 descent		
	Black	Brown	Total
Gastric lesions	198	164	362
Normal	2145	1452	3597
Total	2343	1616	3959

This table presents data on mice of the B1 descent injected with methylcholanthrene at 60 days of age. The mice are classified according to whether they developed gastric lesions or did not develop such a lesion. The mice are further divided into the two color classes, as follows (a) the original color class brown and (b) the mutant black.

led to the conclusion that while one type of tumor susceptibility (fibrosarcomas at the site of injection) is being increased, another type, the gastric lesion, is being decreased. This is evidence that in this particular experimental setup the two cancer susceptibilities are distinct entities even though they both show linkage relationships with the brown tagged chromosome. The exact spatial relationship of the 3 entities or genes on the brown chromosome cannot at present be determined. Another conclusion that seems justified is that in the origin of the original black mutant more than a single point mutation was involved. This finding is of increased significance, since, as far as can be determined now, the original mutation from brown to black itself was a clear cut point mutation, as no variations from typical Mendelian ratios could be determined in the inheritance of mutant black. Skewness to the right of the frequency distribution curve for gastric lesions in brown mice and to the left for the black mutants is suggestive that this may be brought about, in part, by the two cross-over types, a brown mouse with a delayed latent period for a gastric lesion and a black mouse with an enhanced or original early appearance of the gastric lesion. Unfortunately no progeny from these mice were obtained so that this genetic evidence of crossing over could not be obtained.

The present evidence also indicates that

there are specific entities or genes underlying genetic susceptibilities to specific types of neoplasia induced by methylcholanthrene. This interpretation seems to be favored over the alternative concept that in cancer suscep-

tibility, there is a general cancer "gene" that underlies all types of cancer. That is, that there is a gene that determines the difference between the biological states of cancer and not cancer.

16777

Mucolytic Enzyme Systems. IV. Relationship of Hyaluronidase Inhibition by Blood Serum to Incidence of Mammary Cancer in Mice.*

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From previous investigations in this series, it was concluded that the level of hyaluronidase inhibitor in blood serum is elevated in a wide variety of both virus and bacterial infections¹⁻³ as well as in malignant processes.⁴ While these investigations were still in progress Friou and Wenner⁵ reported a similar elevation in rheumatic fever as determined by the mucin clot test, and Thompson and Moses⁶ also found this effect in pneumonia by the clot test. Fulton, Marcus and Robinson⁷ used a method based on decapsulation of a group A hemolytic streptococcus to measure the inhibition by serum and reported no significant

differences between normals, and patients with rheumatoid arthritis.

The inhibiting factor which undergoes change is distinct from the specific antibody inhibitors which are elicited in response to hyaluronidase acting as an antigen. By electrophoresis at pH 8.6 it was found that the non-antibody inhibitor in the serum migrated chiefly with the albumin.⁸ The antibody inhibitor would be expected to be found in the gamma globulin fraction. The factor in question appears to be a non-specific inhibitor since it is capable of inhibiting the enzyme from diverse sources, and furthermore its elevation seems to be a non-specific response to both infection and malignancy. While it is still premature to advance a theory, one might postulate that this response is a defense mechanism designed to counteract the invasiveness potentiated by hyaluronidase. While many organisms do not in themselves possess hyaluronidase, their invasiveness appears to be enhanced, nevertheless, by the presence of the enzyme,⁹ and, accordingly, it is conceivable that a general response against hyaluronidase activity might be employed as a general defense mechanism.

The possibility that hyaluronidase may be involved in the invasive processes of cancer,¹⁰⁻¹⁰ and the great elevation of the serum

* Aided by grants from the Division of Research Grants and Fellowships, National Institute of Health, U. S. Public Health Service, Bethesda, Md., the Medical Research Fund of the Graduate School, University of Minnesota, and the American Cancer Society, upon recommendation of the Committee on Growth of the National Research Council.

1 Glick, D., and Gollan, F., *J. Inf. Dis.*, 1948, **83**, 200.

2 Grais, M. L., and Glick, D., *J. Invest. Dermatol.*, in press.

3 Grais, M. L., and Glick, D., in preparation.

4 Hakanson, E. Y., and Glick, D., *J. Nat. Cancer Inst.*, in press.

5 Friou, G. J., and Wenner, H. A., *J. Inf. Dis.*, 1947, **80**, 185.

6 Thompson, R. T., and Moses, F. E., *Fed. Proc.*, 1948, **7**, 282.

7 Fulton, J. K., Marcus, S., and Robinson, W. D., *Proc. Soc. Am. Bact.*, 1948, **1**, 95.

8 Glick, D., and Moore, D. H., *Arch. Biochem.*, 1948, **19**, 173.

9 Duran-Reynals, F., *Bact. Rev.*, 1942 **6**, 197.

TABLE I.
Hyaluronidase Inhibitor in Blood Serum of Cancer Mice.

Group	Strain	No. mice	Presence of milk agent	Presence of tumor	Mean inhibitor conc. (A) per cc serum	Difference of means	t*	P
1	Breeders	13	—	—	19.2	0.8	.20	>.55
	Ax	11	+	+	18.4			
	Aa							
2	Virgins	22	—	—	16.1	0.2	.05	>.55
	Ax	19	+	—	15.9			
	Aa							
3	Zb	19	—	—	12.8	1.2	.60	>.55
	Zz	14	+	+	11.6			
4	ZbAx F_1	19	—	—	10.1	3.6	1.45	.15
	ZzAa F_1	3	+	+	6.5			

* Statistical quantity used for less than 30 observations to calculate probability (P) that the differences could have resulted from chance alone.

inhibitor in metastatic carcinoma,⁴ led to the present investigation of the level of the inhibitor in the blood serum of various strains of mice in relation to the genesis of mammary cancer. It was hoped that some light might be shed on the question of whether the hyaluronidase inhibitor has any significance in resistance to mammary cancer in mice.

Materials and methods. The mice employed were of the following strains used by Bittner and Huseby.⁷

Aa stock—A stock cancer mice nursed by mothers with "a" milk agent.

Breeders—high cancer line, with cancer when used.

Virgins—low cancer line, without cancer when used.

Ax stock—Decended from 1 animal of the A stock that had been nursed by a female of X or CBA strain. Without cancer when studied. No cancer without milk agent.

Zz stock—Z or C3H stock cancer mice possessing "z" milk agent with cancer when used.

Zb stock—Fostered Z stock mice without milk agent. Descendents from females fostered by mice without milk agent. Without cancer when used.

Zb Ax F_1 —First hybrid generation of Zb \varnothing \times Ax δ . Without cancer when used. Devoid of milk agent.

Zz Aa F_1 —First hybrid generation of Zz \varnothing \times Aa δ . High cancer incidence, both "a" and "z" milk agents present. With cancer when used.

The mice were anesthetized with ether, and 0.5–1.0 cc of blood was taken from the jugular vein of each animal. After clotting, the serum was withdrawn and stored at -25° until used for the determination of hyaluronidase-inhibitor content. The details of the viscosimetric method used, which employed hyaluronidase from bull testes and hylauronic acid from human umbilical cords, have been given in an earlier paper.¹ The value (A) expressing degree of inhibition is defined as $\left(\frac{R-R_0}{R_0}\right)$, where (R₀) equals the time in seconds for the viscosity of the reaction mixture without serum to fall to half its initial value, and (R) equals the corresponding time in seconds for the viscosity to fall to half the initial value in the presence of serum. Only 0.02 cc of serum is required in the 6 cc of total reaction mixture prepared for each measurement. The concentration of inhibitor was calculated as

¹⁰ Boyland, E., and McClean, D., *J. Path. Bact.*, 1935, 41, 560.

¹¹ Pirie, A., *Brit. J. Exp. Path.*, 1942, 23, 277.

¹² Hoffman, D. C., Parker, F., and Walker, T., *Am. J. Path.*, 1931, 7, 523.

¹³ Duran-Reynals, F., *J. Exp. Med.*, 1931, 54, 493.

¹⁴ Duran-Reynals, F., and Claude, A., *Proc. Soc. Exp. Biol. and Med.*, 1934, 32, 67.

¹⁵ McCutcheon, M., and Cowan, D. R., *Cancer Res.*, 1947, 7, 379.

¹⁶ Cowan, D. R., McCutcheon, M., and Zeidman, I., *Cancer Res.*, 1947, 7, 383.

TABLE II.
Relation of Cancer Incidence in Strains of Mice to Hyaluronidase Inhibitor in Blood Serum.

Group	Strain	No. mice	% Cancer ⁷	Mean inhibitor conc. (A) per cc serum	Groups compared	Difference of means	K*	P
1	Aa breeders	13	86.7	15.3	1 and 2	0.5	0.1	>.55
2	Aa virgins	40	3.9	14.8	1 and 3	3.5	1.2	.230
3	Zz breeders	30	95.1	11.8	1 and 4	5.8	2.1	.036
4	ZzAa breeders	21	97.6	9.5	2 and 4	5.3	2.8	.005
					3 and 4	2.3	1.5	.134

* Statistical quantity used for 30 or more observations to calculate probability (P) that the differences could have resulted from chance alone.

(A) per cc serum.

Results and discussion. From the data in Table I it is apparent that within *A* and *Z* strains no significant difference was found between the inhibitor levels in the serums of mice with or without the milk agent, and with or without tumors. The differences in the incidence of cancer between the virgin *A* and *Z* strains appears to result from an "inherited hormonal factor" in the *Z* strain.¹⁷ Bittner and Huseby¹⁷ emphasized the possibility that the inherited susceptibility to mammary cancer may be the same in both strains, while other genes control the "hormonal factor." From the present data it would appear that there is a tendency toward an inverse relationship between the level of the hyaluronidase inhibitor in the serum and the presence of the "inherited hormonal factor". A direct relationship between the porphyrin level and the latter factor was indicated by the work of Bittner and Watson.¹⁸

Since the presence of milk agent or tumor had no appreciable effect on the inhibitor level in the mouse serum, the comparisons in Table II deal only with cancer incidence and the inhibitor. None of the data in Table I could be compared directly with those in Table II because different batches of enzyme and substrate were used to obtain the data for each

table. Variations in the preparations do not justify direct comparisons of results obtained with different lots. A comparison of *A*, *Z*, and their hybrid strains reveals a tendency toward lower inhibitor levels in strains capable of higher cancer incidence as virgins and which bear the "inherited hormonal factor."

Significant differences in the inhibitor levels of Group 2 and 4, and 1 and 4 may be noted in Table II. These differences accompany, in inverse relation, the differences in cancer incidence. Within the *A* strain, large differences in the cancer incidence in breeders and virgins is not associated with a significant difference in the inhibitor level. Furthermore no important differences in the inhibitor are seen between *Aa* and *Zz* strains or the *Zz* and *ZzAa* strains.

Summary. 1. Within the *A*, *Z*, and *AZF*₁ strains of mice, no significant differences in the hyaluronidase-inhibitor levels in serum were found between individuals with and without the milk agent which determines the incidence of mammary cancer. Neither was a significant difference observed between virgins and breeders of the same strain or between individuals with and without tumors. 2. There appeared to be a tendency for the inhibitor titer to vary inversely with the strain incidence of mammary cancer in the *A*, *Z*, and *AZF*₁ mice. 3. A tendency toward an inverse relationship between the inhibitor level and the presence of the "inherited hormonal factor" follows.

¹⁷ Bittner, J. J., and Huseby, R. A., *Cancer Res.*, 1946, 6, 235.

¹⁸ Bittner, J. J., and Watson, C. J., *Cancer Res.*, 1946, 6, 337.

Failure of Estradiol Inactivation Products to Inhibit Pituitary Gonadotrophic Content and Secretion.

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It has been postulated that the level of pituitary gonadotrophins in the blood is regulated principally by the degree of activity of the ovaries, functioning ovaries inactivating gonadotrophins, and non-functioning ovaries permitting gonadotrophins to accumulate in the circulation.¹ This hypothesis was based on observations of female rats with ovaries auto-transplanted to the spleen. By this means, ovarian secretion was directed through the portal vein directly to the liver, where the estrogenic substances were inactivated sufficiently to prevent their detection in the systemic circulation. As in castrates, it was noted that vaginal cell atrophy, uterine atrophy and thymic hypertrophy occurred; however, pituitary gonadotrophic content did not rise, as in castrates, but remained at normal levels. This refuted the concept that normal levels of estrogen maintain normal pituitary gonadotrophic content, and suggested instead that pituitary gonadotrophic content is the resultant of the activity of functional ovaries upon circulating gonadotrophins.

An alternate explanation for the lack of rise of pituitary gonadotrophins is the possible influence of estrogen inactivation products upon the hypophysis. In animals with their ovaries transplanted to the spleen, degradation products of the estrogens which have passed through the liver, although lacking estrogenic activity, might conceivably depress the hypophysis.

Smith² found that Westerfeld's lactone, an inactivation product of estrone, increased pituitary content of gonadotrophins. Segaloff³

has found that bisdehydrodoisynolic acid, a degradation product of estrogens, is more active when administered intrasplenically than when injected subcutaneously. Since some inactivation products of estrogens do have effects on pituitary gonadotrophic content, it seemed possible that they might be the principal controlling factor. This possibility is the subject of the present investigation.

Only estrone and estradiol have been recovered from ovarian tissue. As estrone is changed to estradiol in the process of its inactivation by the liver,⁴ α -estradiol was chosen as the estrogen for this study.

Materials and methods. Adult virgin female rats of the Sprague-Dawley strain weighing 200-250 g were castrated, and on the same day α -estradiol pellets weighing 1.1 to 10.8 mg were implanted in their spleens. One group of rats received pellets subcutaneously, and another group was included as intact controls. Operated rats with pellets implanted in the spleen which spontaneously developed vascular adhesions from the spleen to the systemic circulation served as additional controls.

From 33 to 40 days after the pellets were implanted, the donor rats were killed by decapitation. Pituitary gonadotrophin content was measured by suspending each anterior pituitary gland in 6.0 cc of saline by repeatedly drawing into and expelling from a syringe, then by injecting into one 24-day-old Sprague-Dawley female rat, 1.0 cc twice daily for three days. The assay rats were killed 24 hours after the last injection. Results are tabulated in Table I.

Discussion. The control of pituitary gonadotrophic secretion could conceivably be

* Schering Fellow in endocrinology.

¹ Jungck, E. C., Heller, C. G., and Nelson, W. O., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 148.

² Smith, O. W., *Proc. Soc. Exp. Biol. and Med.*, 1945, **50**, 242.

³ Segaloff, A., *Fed. Proc.*, 1947, **6**, 399.

⁴ Heller, C. G., *Endocrin.*, 1940, **26**, 619.

TABLE I.

Donor rats										Recipient rats			
No. of rats	Vaginal smears		α-estradiol pellets				At autopsy*		Uterus		Ovarian wt, mg	No. of rats	
	Before operation	After operation	Avg wt when implanted, mg	Avg absorbed, μg/day	Appearance of uterus	Thymic wt, mg	With fluid, mg	Without fluid, mg					
Intact controls	3	cycling			—	—	normal	183	185	111	20	3†	
Castrate controls	11	"			—	—	atrophic	241	95	95	103	11†	
α-estradiol pellet in spleen, no adhesions	16	"			5.4	21.9	"	293	115	115	78	16	
Same	6	"			(1.1-10.2)	(5.4-47.5)							
with adhesions		"		estrus	5.3	19.7	estrus	83	54	54	95	6	
α-estradiol pellet SQ	3	"		"	(2.3-10.8)	(8.8-32.5)	"	38	34	34	16	3	
					6.2	74.0							
					(4.3-9.7)	(39.4-119.0)							
Uninjected recipient rats										36	13	63	
* Autopsy 33-40 days after implantation of pellets													

* Autopsy 23-40 days after implantation of pellets.

† These values are in keeping with previous observations made on the same strain of rats.

due to estrogen inactivation products from the liver. Thus, when an animal is castrated, there are no estrogen inactivation products and the pituitary is permitted to increase markedly in gonadotrophic content. When the ovaries are present in the spleen, the pituitary gonadotrophic content would continue to be controlled by the estrogen inactivation products, thus accounting for the normal gonadotrophin levels found in these animals.

To test this hypothesis, pellets of α-estradiol were implanted into the spleens of mature female rats castrated the same day. The relationships in the experimental rats are diagrammed in Fig. 1. An α-estradiol pellet (at 1 in the figure) was implanted in the spleen. The estradiol was carried directly to the liver (at 2) and inactivated, so no active estradiol reached the general systemic circulation. (This was confirmed by the castration response elicited — hypertrophied thymus, atrophic uterus and atrophic vaginal smear.) Inactivation products of estradiol arrived in the circulation and were free to act on hypophyseal gonadotrophic content and secretion (at 3).

If the principal controlling factor of pituitary gonadotrophic content is estrogen inactivation products, then castrated female rats having pellets of α-estradiol in their spleens should have normal pituitary gonadotrophin content. If the main controlling mechanism is inactivation of gonadotrophins by the ovaries, as postulated, then the presence of estradiol pellets in the spleen should have little effect in maintaining normal pituitary gonadotrophic potency of castrated female rats.

The pituitaries of normal unoperated female rats contained sufficient gonadotrophin to stimulate recipient ovarian weights from 13 mg to 20 mg. Castrate control rat pituitaries stimulated recipient ovarian weight from 13 mg to 103 mg. The animals with α-estradiol pellets in the spleen (and no adhesions) had sufficient gonadotrophin in their pituitaries to stimulate recipient ovarian weight from 13 mg to 78 mg, which is in the castrate range. The average daily absorption of α-estradiol was 21.9 μg, several times the

ESTROGEN-GONADOTROPHIC RELATIONSHIP

ESTRADIOL PELLETS IMPLANTED INTO SPLEEN

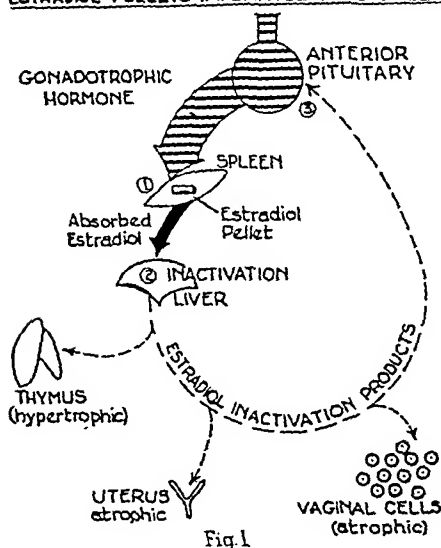


Fig. 1

dose necessary to maintain constant estrus in the intact animal. This large dose of α -estradiol was completely inactivated, as demonstrated by the atrophic uteri and vaginal smears, and the large thymus. Therefore, many times the physiological amount of

inactivation products were present in the circulation. However, the pituitary gonadotrophic content rose to castrate levels.

The castrated rats with pellets implanted in the spleen which developed vascular adhesions from the spleen to the systemic circulation absorbed essentially the same amount of α -estradiol daily as the rats without adhesions ($19.7 \mu\text{g}$ vs. $21.9 \mu\text{g}$). The rats with estradiol pellets implanted subcutaneously absorbed $74.0 \mu\text{g}$ daily. In both the adhesion group and in the rats with pellets implanted subcutaneously, constant estrus was observed along with atrophy of the thymus and suppression of pituitary gonadotrophic content. These changes are noted only with unphysiologically large doses of estrogen,¹ thus indicating that larger than normal amounts of estrogen were being absorbed.

Conclusions. 1. The inactivation products of α -estradiol produced by passage of estradiol through the liver do not exert a significant inhibiting influence on anterior pituitary gonadotrophic secretion.

2. The absence of this effect supports the thesis that pituitary gonadotrophic content and secretion are controlled principally by ovarian inactivation of circulating gonadotrophins.

16779

Effect of Adrenal Cortical Extract on Recovery from Severe Pneumococcal Infection in Mice.*

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Dallas, Texas.

Many observations suggest that the increased adrenal cortical secretion during stress provides an increase in nonspecific resistance.¹ In patients or animals moribund from infections, it was thought that administration of

* This study was supported by grants from the United States Public Health Service, and from the Lederle Laboratories Division, American Cyanamid Company.

¹ Selye, H., *J. Clin. Endocrinol.*, 1946, 6, 117.

large doses of adrenal cortical extract might temporarily increase resistance and thus gain time for antibacterial agents such as penicillin to effect recovery. To test this possibility two similar experiments were performed.

Methods. Albino Swiss mice were inoculated intraperitoneally with large numbers of Type I pneumococci. After a certain percentage of the inoculated mice had died, the survivors were placed at random into 3 dif-

CORTIN THERAPY IN PNEUMOCOCCUS INFECTION

TABLE I.
Effect of Adrenal Cortical Extract and Penicillin on Survival of Mice Infected with *Pneumococci*.
Treatment begun 22 hours after inoculation when 5% of mice were dead.

Hr after pneumococcus inoculation	Group I Penicillin G + adrenal cortical extr.		Group II Penicillin G + corn oil		Group III Saline + corn oil	
	No. living	% living	No. living	% living	No. living	% living
22	34	100	34	100	25	100
24	18	53	23	68	15	60
26	15	44	16	47	4	16
28	13	38	13	38	1	4
30	12	35	10	29	0	0
34	10	29	9	26	0	0
38	9	26	8	24	0	0
42	8	24	8	24	0	0
46	8	24	8	24	0	0
50	7	21	8	24	0	0

TABLE II.
Effect of Adrenal Cortical Extract and Penicillin on Survival of Mice Infected with *Pneumococci*.
Treatment begun 22 hours after inoculation when 10% of mice were dead.

Hr after pneumococcus inoculation	Group I Penicillin G + adrenal cortical extr.		Group II Penicillin G + corn oil		Group III Saline + corn oil	
	No. living	% living	No. living	% living	No. living	% living
22	37	100	39	100	14	100
24	30	81	35	90	14	100
26	26	70	31	80	13	93
28	22	59	30	77	11	79
30	22	59	30	77	8	57
34	21	57	29	74	3	21
38	21	57	28	72	3	21
42	20	54	28	72	0	0
46	20	54	28	72	0	0
50	19	51	25	64	0	0

ferent therapy groups.

Group I received 250 units of crystalline sodium penicillin G dissolved in 0.5 cc saline intraperitoneally every 2 hours. These mice were also injected subcutaneously with 0.2 cc of adrenal cortical extract (Upjohn's "Lipo-Adrenal Cortex") immediately after the first penicillin injection. Subsequently, at 4 and 8 hours after this injection they received an additional subcutaneous injection of 0.1 cc of adrenal cortical extract.

Group II was treated on the same dosage schedule of penicillin as Group I. However, subcutaneous injections of corn oil (Mazola) were administered instead of adrenal cortical extract.

Group III was a control group. Mice in

this group received 0.5 cc of saline instead of the penicillin solution every 2 hours, as well as the same type of corn oil injections received by Group II. In both experiments the therapeutic program was carried out for 28 hours.

First experiment. Ninety-eight mice averaging 14 g in weight were inoculated intraperitoneally with 25,000 pneumococci per mouse. Treatment was delayed until 22 hours after inoculation when 5% of the mice were dead. The 93 survivors were divided at random into 3 groups and treated according to the plan described above.

Second experiment. One hundred mice averaging 20 g each were inoculated intraperitoneally with 75,000 pneumococci per

mouse. Treatment was withheld until 22 hours after inoculation when 10% of the animals had died. The 90 survivors were divided at random into 3 treatment groups as outlined above.

In addition, control experiments were performed which showed that adrenal cortical extract, corn oil, penicillin and saline injections in the dosages and schedules used in the experiments had no noticeably harmful effects on uninfected mice.

Results and comments. The mortality in the 3 treatment groups is indicated in Table I for the first experiment and in Table II for the second experiment. As would be expected, the groups treated with penicillin showed a much lower mortality than the controls receiving no penicillin. No further improvement in mortality was effected by additional treatment with large doses of potent adrenal

cortical extract. If these experiments are applicable to clinical situations, the results do not lend encouragement to the use of adrenal cortical extracts in the treatment of patients moribund from bacterial infections.

Summary. Mice of varying weights were inoculated intraperitoneally with large numbers of Type I pneumococci and the infections allowed to proceed until from 5 to 10% of the animals had succumbed. The survivors were divided into groups at random and treated with penicillin and penicillin plus potent adrenal cortical extract. No improvement was obtained in the therapeutic results achieved with penicillin by the additional treatment with adrenal cortical extract.

We wish to thank Dr. E. Gifford Upjohn of the Upjohn Pharmaceutical Company for generously supplying us with Lipo-Adrenal Cortex (Upjohn).

16780

Reproduction of Human Ulcerative Pulmonary Tuberculosis in Rabbits by Quantitative Natural Airborne Contagion.*

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The purpose of this report is to outline a study in which the acquisition and development of human ulcerative pulmonary tuberculosis was closely simulated in rabbits by exposing them to the inhalation of known numbers of tubercle bacilli while breathing naturally in an experimental apparatus.

Materials and methods. An apparatus for airborne infection of rabbits, constructed at the Cornell University Medical School, was placed at our disposal by Dr. Eugene L. Opie. This was modified in accordance with the principles elaborated by Wells¹ and further

adjusted by certain appliances directed toward improving its quantitative aspects.

Fig. 1 gives a schematic drawing of the instrument. Briefly, a fine suspension of largely isolated, virulent bovine type tubercle bacilli, freed from clumps larger than a red blood cell by filtration through Whatman number 5 filter paper, is atomized with a known volume of compressed air in unit time through a specially designed nozzle. The large droplets settle out quickly in the spraying flask. A uniform flow of droplet nuclei, containing tubercle bacilli, is delivered into a mixing device where it is diluted with room air. Thence the contaminated air is drawn through a 16-foot pipe into a chamber where the rabbits are exposed. This chamber is exhausted by the draft action of a hot flame at the bottom of a baffled chimney connected

* Aided by grants from the Commonwealth Fund and the Faculty Research Committee, University of Pennsylvania.

† Tuberculosis Control Division, Public Health Service, Federal Security Agency.

¹ Wells, W. F., *Science*, 1940, 91, 172.

APPARATUS FOR QUANTITATIVE AIRBORNE INFECTION (1944-1946)

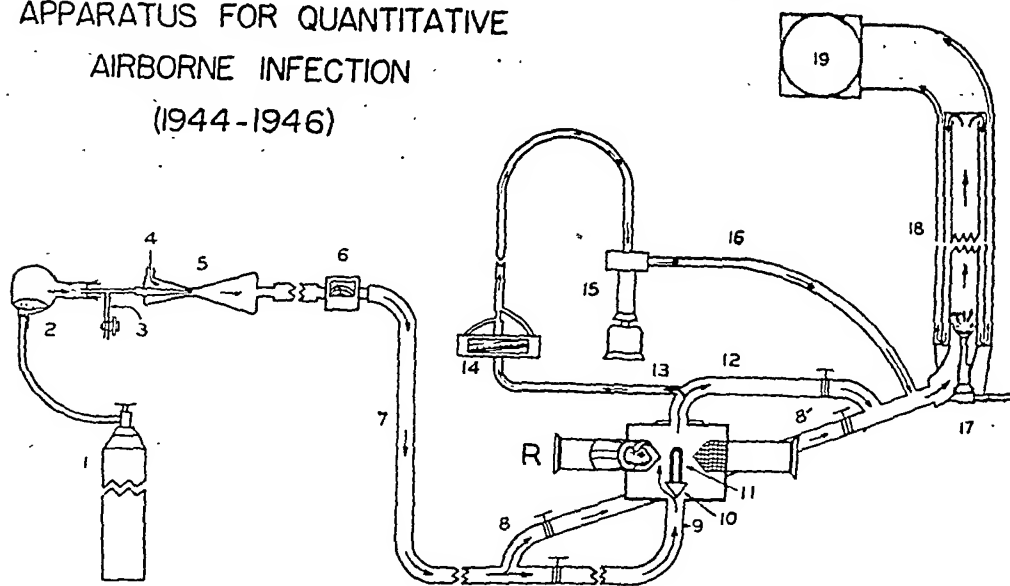


FIG. 1.

1—Compressed air tank. 2—Atomizer and spraying flask. 3—Outlet for sampling aerosol. 4—Air inflow from room. 5—Mixing device. 6—Flow-meter (liters/min.). 7—Tube which conveys infected air. 8-8'—Shunt. 9—Inlet tube to exposure chamber. 10—Spreading cone. R—Rabbit in cylinder, with head protruding into exposure chamber through rubber collar about its neck. 11—Ultraviolet lamp. 12—Outlet tube from exposure chamber. 13—Outlet for sampling exposure chamber. 14—Inclined draft gauge. 15—Wells air centrifuge. 16—Air centrifuge exhaust tube. 17—Burner. 18—Baffled incinerating chimney. 19—Exhaust fan.

to the outlet of the chamber. The incinerated air is exhausted to the outdoor atmosphere by a fan.

The concentration of tubercle bacilli in the air respired by the exposed rabbits is determined culturally² with the aid of a Wells air centrifuge, provided with a calibrated inclined draft gauge, which gives the exact volume of air sampled. Since the total volume of contaminated air respired by the rabbits during their period of exposure can be determined,³ the number of bacilli to which they were exposed can be calculated.

If the bacilli in the exposure chamber are too few to be accurately determined directly, they can be estimated by ascertaining their number in the aerosol immediately after it leaves the spraying flask and before it is diluted by the inflowing room air. The dilution factor existing between the concentration of bacilli in this aerosol and that in the exposure chamber can be empirically determined

by numerous preliminary calibrations. This factor depends, in part, on the volume of room air added to a unit volume of aerosol in a given time, and can be calculated from data obtained by a flowmeter which measures the rate of air flow through the system. This determination is made possible by introducing a shunt into the duct which feeds the infected air to the exposure chamber. By means of this shunt, which is provided with appropriate valves, the infected air can be made to bypass the exposure chamber in which the rabbits are placed, and in which they can breathe uninfected air while the concentration of bacilli in the aerosol is ascertained. At the desired moment, without altering the inflow of infected air and without danger to the operators, the valves can be adjusted and the contaminated air allowed to flow through the exposure chamber.

Experimental. Twenty-nine rabbits, of unknown genetic resistance to tuberculosis, in groups of 2 to 5, in 9 separate experiments were exposed to the inhalation of varying numbers of highly virulent bovine type tuber-

² Lurie, M. B., *J. Exp. Med.*, 1934, 60, 163.

³ Murphy, D. C., and Thorpe, E. S., *J. Clin. Invest.*, 1931, 10, 545.

TABLE I.
Relation Between the Number of Tubercle Bacilli Respired and the Number of Tubercles Developed.

1	2	3	4	5	6
Exp. No.	No. of tubercle bacilli per l. respired air	Rabbit No.	No. of tubercle bacilli calculated as respired	No. of primary tubercles found in both lungs	Ratio between No. of tubercle bacilli respired and No. of tubercles in lungs
1	1217	1	8,819	591	14.9
	"	2	8,041	973	8.2
	"	3	10,116	1,270	8.0
2	643	4	3,858	1,344	2.8
	"	5	4,629	833	5.5
3	468	6	2,808	425	6.6
	"	7	2,574	605	4.2
	"	8	2,808	397	7.1
	"	9	2,808	387	7.2
4	228	10	1,436	574	2.5
	"	11	1,276	397	3.2
	"	12	1,413	1,012 tubercle bacilli recovered from lungs	
5	91	13	627	59	10.6
	"	14	664	82	8.1
	"	15	700	38	18.4
	"	16	664	48	13.8
	"	17	609	104	5.8
6	87	18	522	130	4.0
	"	19	609	59	10.3
	"	20	600	105	5.7
7	17	21	114	5	23.0
	"	22	122	15	8.1
8	13	23	78	18	4.3
	"	24	78	39	2.0
	"	25	74	16	4.6
	"	26	75	100 tubercle bacilli recovered from lungs	
9	5	27	32	3	10.1
	"	28	32	1	32.0
	"	29	31	0	—
Arg					9 ± 7

cle bacilli of the strain Ravenel grown on a modified Lowenstein medium.² The number of bacillary units in the air respired by the rabbits in the individual experiments ranged from 5 to over 1200 per liter, and are listed in column 2, Table I.

Given the duration of exposure of each rabbit to the known concentration of bacilli in its respired air, the total number of bacillary units to which the rabbits were exposed can be calculated by Kleiber's formula.⁴ This

states that animals inhale 212 cc of air per minute per kilo of body weight to the three-quarter power in order to satisfy their oxygen needs. Column 4 lists these figures.

That the number of bacilli calculated to have been inhaled by this method is not far from the actual number of microorganisms found in the lung immediately after exposure has been noted previously.⁵ It is also suggested in this table. It will be seen that in

⁵ Wells, W. F., and Lurie, M. B., *Am. J. Hyg.*, 1941, 34, Sect. B, 21.

⁴ Kleiber, M., *Science*, 1944, 99, 542.

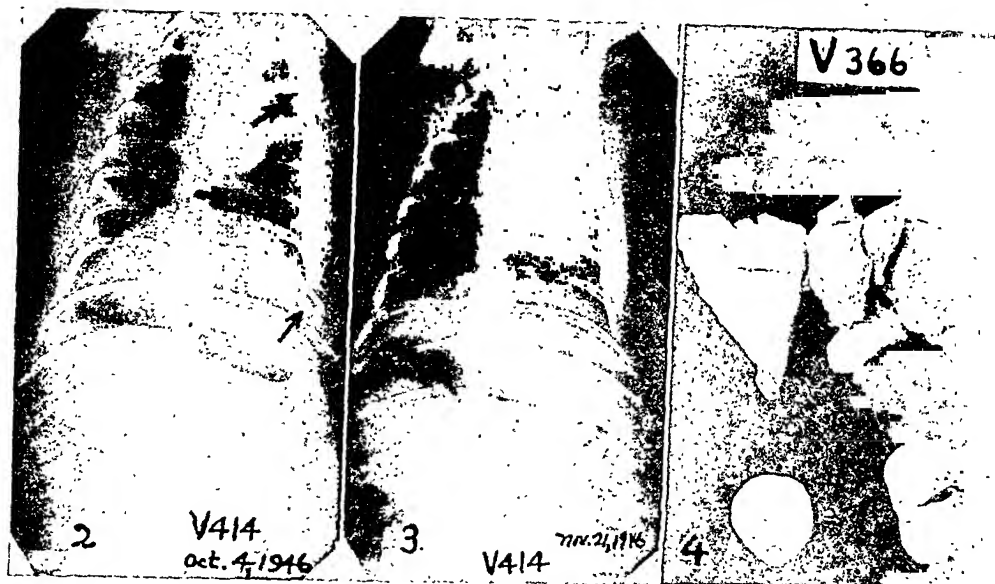


FIG. 2. Radiograph of rabbit V 414, 67 days after exposure. Three foci are visible. A walled off cavity in the third interspace and areas of consolidation in the sixth and eighth interspaces, respectively, of the left lung are indicated by arrows.

FIG. 3. Radiograph of the rabbit shown in Fig. 2, 48 days later. The progression of the disease in the left lung is evident. The right lung is normal.

FIG. 4. The lungs and kidneys of rabbit V 366 at death, 9.5 months after exposure. One of the 3 primary cavities found in the right lung is seen in the lower lobe. Infarcts of nontuberculous origin in both kidneys.

experiment 4 it was estimated that rabbit 12 had inhaled 1413 bacillary units. Actually, 1012 tubercle bacilli were recovered from its lungs immediately after exposure, as recorded in column 5. Again, in experiment 8, it was calculated that rabbit 26 had inhaled 75 bacilli. Actually, column 5 shows that 100 organisms were cultured from its lungs. Similarly close checks between the bacilli calculated as inhaled and the number recovered from the lungs have been observed since on many occasions.

Five to 6 weeks after exposure the rabbits were killed and the number of primary tubercles present in their lungs was carefully determined by excising each individual focus in both lungs. In those instances where the tubercles were too numerous for direct counting, these were estimated as follows: After removing the bronchi at their junction with the pulmonary parenchyma, both lungs were weighed. One lobe from each lung was also accurately weighed and the number of tubercles in these directly counted by enucleation of each nodule. By simple ratio the number

of tubercles present in both lungs was calculated. This procedure was justified because, in these instances, the tubercles were uniformly distributed throughout both lungs.

It will be noted in columns 4 and 5 that there is a general correlation between the number of primary tubercles found in the lungs and the number of bacillary units estimated to have been inhaled. The larger the number inhaled the larger the number of primary pulmonary foci. However, there is no constant ratio between the number of bacilli required to generate a single tubercle in the different experiments, nor even in different rabbits of the same experiment inhaling the same infected air. This ratio is listed in column 6, and ranged from 2 to 32 bacillary units per tubercle in the 9 experiments. In one and the same experiment this ratio was often 3 times greater in one animal than in another. The average ratio in all the 26 rabbits was 9 ± 7 bacillary units per tubercle generated. Whether this ratio depends on the native resistance of rabbits or on other factors has not yet been determined.

TABLE II.

Fate of Rabbits of Race III, Sensitized with Heat-killed Tubercle Bacilli and Exposed 5.5 Months Later to the Inhalation of About 50 Virulent Bovine Tubercle Bacilli.

Rabbit No.	Survival after exposure, mo.	Type of tuberculosis
V 369*	Still living, 26	No evidence of tuberculosis.
U 784	Killed, 4.7	No tuberculosis.
V 366	9.5	3 small, well walled off cavities in right lung. No tuberculosis elsewhere, including hilum nodes. Large infarcts in both kidneys.
V 30	10.1	1 large cavity with limited bronchogenic spread in upper lobe of each lung. Hilum nodes normal. Ulcerative tuberculosis of larynx. Single miliary tubercle in one kidney.
V 414	7.3	Unilateral ulcerative pulmonary tuberculosis with slight, contralateral lesions. Hilum nodes normal. Miliary tubercles in each kidney. Ulcerative laryngeal tuberculosis. One large tuberculous pleural nodule. Tuberculosis of one wrist joint.
V 267	6.6	Completely excavated tuberculosis of all lobes of right lung, including the azygous lobe. Consolidation of upper lobe of left lung and bronchogenic spread to lower lobe of same lung. Hilum nodes normal. Few miliary tubercles in one kidney.

* This rabbit was not sensitized with heat-killed tubercle bacilli before exposure.

Using the above observations as a basis, a group of 5 highly inbred rabbits of Race III, obtained from Dr. Paul B. Sawin of the Roscoe B. Jackson Memorial Laboratory, were given 6 consecutive, weekly intracutaneous injections of heat-killed bovine type tubercle bacilli, totaling 7 mg per rabbit. Five and one-half months after the last injection of heat-killed tubercle bacilli, these 5 vaccinated rabbits together with a sixth unvaccinated animal of the same race were exposed simultaneously in the apparatus for 10 minutes to droplet nuclei of virulent bovine type tubercle bacilli of the Ravenel strain derived from a culture on glycerol agar. There were 10 bacillary units per liter in the air respired by these rabbits. On the basis of the above noted Kleiber formula it was estimated that each rabbit inhaled about 5 liters of this infected air, or about 50 bacilli. Two months after exposure some of the rabbits showed radiographic evidence of sharply defined pulmonary lesions, some with cavity formation (Fig. 2); 2-3 such foci were found in these rabbits at this time. It must be noted that preliminary studies on this inbred race of rabbits had shown that they were of high genetic resistance to tuberculosis.

Table II details the results of this experi-

ment. It will be noted that the unvaccinated rabbit, V 369, had never shown any evidence of tuberculosis and is still living 26 months after the exposure. A vaccinated animal, U 784, was killed 4.7 months after exposure and no gross tuberculosis was found anywhere in the body. The remaining 4 vaccinated rabbits showed strictly localized ulcerative pulmonary tuberculosis of varying degree of extension. In no instance were the draining tracheobronchial lymph nodes involved, and hematogenous dissemination to the rest of the body was limited to a few scarcely visible miliary tubercles in the cortex of one or both kidneys, with the single exception stated below.

The least extensive disease was seen in rabbit V 366 (Fig. 4). In its right lung there were three small, well walled off cavities, about 2-5 mm in diameter, containing tubercle bacilli. These apparently represent the original primary foci which had undergone caseation and liquefaction and had become encapsulated. There was no bronchogenic, lymphogenous, or hematogenous dissemination beyond these primary lesions when the rabbit died from renal infarction 9.5 months after infection as a result of a surgical operation.

In V 30 (Fig. 5) the disease spread by

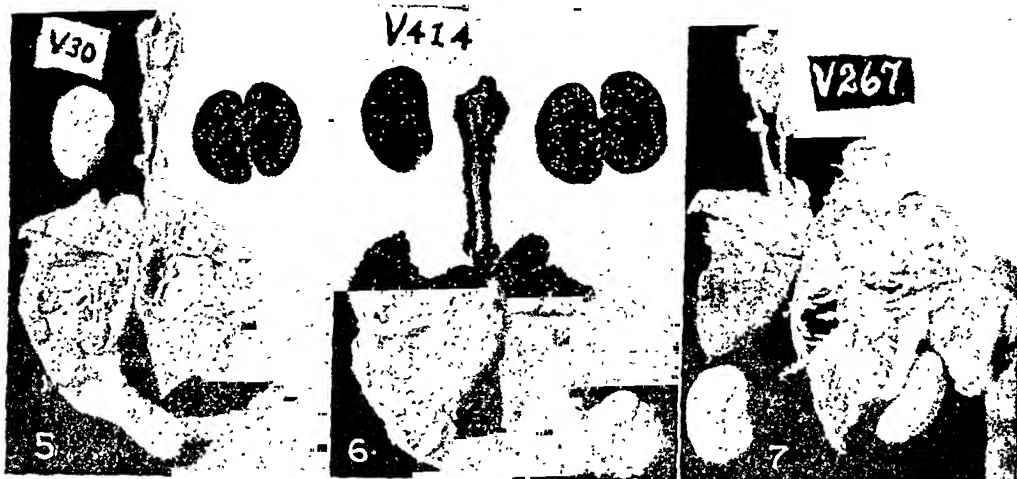


FIG. 5. The organs of rabbit V 30 at death, 10.1 months after exposure. One large cavity in each lung with limited bronchogenic spread in both. Tracheobronchial nodes and kidneys, normal. Ulcerative tuberculous laryngitis with tubular spread to the appendix, seen below left lung.

FIG. 6. Organs of rabbit V 414 at death, 7.3 months after exposure. Unilateral ulcerative tuberculous in left lung with slight bronchogenic dissemination in the upper lobe of right lung. Tracheobronchial nodes and kidneys, normal. Ulcerative tuberculous laryngitis. Tuberculous pleural nodule in the right lower corner of photograph. The progression of the disease in this rabbit during the first 4 months of the infection is depicted in Figs. 2 and 3 above.

FIG. 7. The organs of rabbit V 267 at death, 6.6 months after exposure. Completely excavated tuberculous of all lobes of right lung, including azygous lobe which is depicted just above the right kidney. Consolidation of upper lobe of left lung and bronchogenic spread to lower lobe of the same lung. Tracheobronchial nodes, normal. The few, minute, miliary tubercles in the kidneys cannot be seen.

bronchogenic dissemination to a considerable portion of both lungs from a single large cavity in each. The rabbit was asphyxiated by an ulcerative tuberculous laryngitis 10.1 months after infection. In the next rabbit of this series, V 414, the disease extended to a greater degree. Fig. 2 and 3 show the progression of the disease in the lungs during the first 4 months of the infection. Fig. 6 illustrates the character of the tuberculosis at death. There was a unilateral destructive phthisis with slight contralateral lesions. This rabbit also died with tuberculous, ulcerative laryngitis, 7.3 months after its infection. There was some pleural tuberculosis and one wrist joint was involved. Finally, the disease extended most rapidly in rabbit V 267, which died 6.6 months after exposure to the inhalation of 50 tubercle bacilli from complete excavation of all the lobes of the right lung including the azygous lobe. Fig. 7 illustrates the resulting thick walled cavities, the caseous consolidation of the upper lobe of the oppo-

site lung and the bronchogenic spread to the lower lobe of the same lung.

Thus, a group of 6 rabbits of the same highly inbred, genetically resistant family showed all the degrees of resistance as seen in man; from failure of the disease to take root at all, to the formation of primary lesions which did not extend beyond their site of inception, to limited bronchogenic dissemination from the primary ulcerative foci, to unilateral ulcerative phthisis, and finally, to a rapidly progressive ulcerative tuberculosis which had destroyed one lung and was progressing from the upper to the lower lobes in the contralateral lung. The similarity of these observations to the varying types of ulcerative pulmonary phthisis as seen in man is striking.

While these animals all showed a localization of the disease which is characteristic of resistant rabbits, as evidenced by their capacity to limit the infection to the portal of entry, the lung,⁶ they varied markedly in

their ability to restrict the dissemination of the disease by bronchogenic spread.

It is noteworthy that there was evidence in some of the rabbits that 50 inhaled bacilli had given rise to about 3 primary pulmonary lesions. These 3 pulmonary lesions were seen in rabbit V 366 at death and, in rabbit V 414 (Fig. 2), 3 foci were identified in the radiograph of its lungs 2 months after infection. It is noted above that an average of 9 ± 7 tubercle bacilli were necessary to generate a single tubercle in the preliminary experiments. Therefore, taking the larger figure, 16, as applicable in this series, 3 primary pulmonary foci would be expected from these 50 inhaled bacilli. The larger ratio is likely for this group since these rabbits, unlike the former animals, were slightly immunized with heat-killed tubercle bacilli prior to exposure.

Summary. A modification of an apparatus for quantitative airborne infection as devised by Wells is described. Its use has indicated that, while the number of tubercles generated in the lung is to a certain degree proportional

to the number of droplet nuclei of tubercle bacilli inhaled, there is no constant ratio between the number of tubercle bacilli arrested in the lung and the number of primary tubercles generated therein, even when the rabbits breathe the same infected air simultaneously.

By exposing vaccinated highly inbred, genetically resistant rabbits to the inhalation of known small numbers of virulent bovine type tubercle bacilli it was possible to reproduce various types and phases of human localized ulcerative pulmonary tuberculosis of the adult or reinfection type. These phases ranged from complete resistance to the infection to varying degrees of bronchogenic dissemination from excavated foci to one or both lungs. There was little or no lymphogenous or hematogenous dissemination from the pulmonary portal of entry in these rabbits.

It is a pleasure to acknowledge the aid given us by Mr. R. J. Ott of the Philadelphia Gas Co., in calibrating the inclined draft gauge, and that of Mr. Peter Zappasodi for the photographs in this paper.

⁶Lurie, M. B., *Am. Rev. Tuberc.*, 1941, **44**, Suppl. 1.

16781

Failure of Antihistaminic Drugs to Reduce Reactive Hyperemia in Man.*

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The physiological response of regional dilatation consequent to circulatory arrest has been designated as reactive hyperemia. The studies of Lewis and Grant¹ and Goldblatt² presented evidence indicative that this is a

local response and the former suggested that the accumulation of a chemical was responsible for the vasodilatation. Later Lewis³ labeled this agent H-substance and defined it as "any substance (or substances) that is liberated by the tissue cells and exerts on the minute vessels and nerve endings an influence culminating in the triple response". Subsequently others⁴⁻⁶ reported finding increased amounts

* Aided by a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

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¹ Lewis, T., and Grant, R., *Heart*, 1925-26, **12**, 73.

² Goldblatt, H., *Heart*, 1925-26, **12**, 281.

³ Lewis, T., *The Blood Vessels of the Human Skin and Their Responses*, 1927, London, Shaw and Sons.

⁴ Barsoum, G. S., and Gaddum, J. H., *J. Physiol.*, 1935, **85**, 13P.

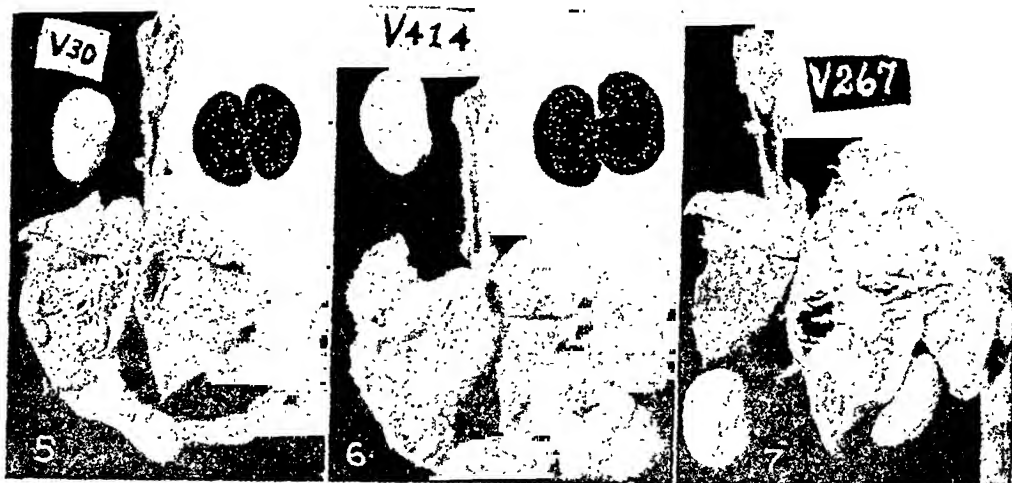


FIG. 5. The organs of rabbit V 30 at death, 10.1 months after exposure. One large cavity in each lung with limited bronchogenic spread in both. Tracheobronchial nodes and kidneys, normal. Ulcerative tuberculous laryngitis with tubular spread to the appendix, seen below left lung.

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FIG. 7. The organs of rabbit V 267 at death, 6.6 months after exposure. Completely excavated tuberculosis of all lobes of right lung, including azygous lobe which is depicted just above the right kidney. Consolidation of upper lobe of left lung and bronchogenic spread to lower lobe of the same lung. Tracheobronchial nodes, normal. The few, minute, miliary tubercles in the kidneys cannot be seen.

bronchogenic dissemination to a considerable portion of both lungs from a single large cavity in each. The rabbit was asphyxiated by an ulcerative tuberculous laryngitis 10.1 months after infection. In the next rabbit of this series, V 414, the disease extended to a greater degree. Fig. 2 and 3 show the progression of the disease in the lungs during the first 4 months of the infection. Fig. 6 illustrates the character of the tuberculosis at death. There was a unilateral destructive phthisis with slight contralateral lesions. This rabbit also died with tuberculous, ulcerative laryngitis, 7.3 months after its infection. There was some pleural tuberculosis and one wrist joint was involved. Finally, the disease extended most rapidly in rabbit V 267, which died 6.6 months after exposure to the inhalation of 50 tubercle bacilli from complete excavation of all the lobes of the right lung including the azygous lobe. Fig. 7 illustrates the resulting thick walled cavities, the caseous consolidation of the upper lobe of the oppo-

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Thus, a group of 6 rabbits of the same highly inbred, genetically resistant family showed all the degrees of resistance as seen in man; from failure of the disease to take root at all, to the formation of primary lesions which did not extend beyond their site of inception, to limited bronchogenic dissemination from the primary ulcerative foci, to unilateral ulcerative phthisis, and finally, to a rapidly progressive ulcerative tuberculosis which had destroyed one lung and was progressing from the upper to the lower lobes in the contralateral lung. The similarity of these observations to the varying types of ulcerative pulmonary phthisis as seen in man is striking.

While these animals all showed a localization of the disease which is characteristic of resistant rabbits, as evidenced by their capacity to limit the infection to the portal of entry, the lung,⁶ they varied markedly in

TABLE I.
Effect of Antihistaminic Drug Upon Blood Flow in the Foot-Leg at Rest and During Reactive Hyperemia.

Subject and drug*	Blood flow cc/min./100 cc limb (Each value is the average of the number of measurements indicated in parentheses)						
	Initial inflow after release of occlusions maintained for						
	Resting flow	30 sec.	1 min.	2 min.	3 min.	4 min.	5 min.
Pr. before after 50 mg B	3.00 (20)	5.7 (3)	6.8 (3)	7.8 (1)	8.7 (2)		
	2.41 (22)	5.5 (2)	7.7 (1)	8.5 (1)	9.4 (1)		
Hu. before (right) after 50 mg P†	0.78 (21)						13.1 (2)
	1.33 (22)					12.0† (1)	12.9 (2)
Hu. before (left)‡ after 20 mg B	6.56 (14)	15.0 (1)	12.4 (2)		12.8 (1)		
	6.04 (7)	12.8 (2)	14.5 (3)		13.8 (1)		
Be. before after 20 mg B	4.43 (8)						8.6 (1)†
	3.53 (9)						10.2 (1)
Bo. before after 20 mg B	0.76 (28)		3.1 (2)	5.3 (2)	6.3 (2)		10.5 (3)
	0.75 (19)		5.3 (2)	5.7 (1)	7.9 (1)		
Jo. before after 30 mg B	1.35 (30)	2.0 (2)	4.0 (2)	5.4 (2)	5.5 (2)		
	1.21 (9)	2.3 (4)	5.3 (2)	6.1 (2)	6.0 (2)		
Cl. before after 30 mg B	1.45 (13)	2.4 (2)	3.2 (1)	6.3† (2)	8.4 (2)		
	3.10 (11)	3.3 (2)	4.9 (3)		8.0 (1)		
Average before after	2.70	6.29	5.91	6.14	8.31		13.1
	2.62	5.98	7.54	6.77	9.00		12.9

* B = intravenous benzydyl; P = oral pyribenzamine.

† Not included in average.

‡ Sympathectomized.

of histamine, or of a substance with the "biological properties of histamine", in the venous blood during reactive hyperemia and concluded that this was responsible, at least in part, for the vasodilatation. Emmelin *et al.*⁷ and Kwiatkowski,⁸ using the same or modifications of the same method of analysis for histamine, were unable to confirm these results. They stated, however, that their findings did not exclude the possibility that histamine is formed *in situ* and plays a role in the development of reactive hyperemia, as the possibility remains that it might not readily diffuse away from the site of action.

Despite certain objections,⁹ Lewis' studies and the subsequent work have led to the generally accepted hypothesis that the vascular response in reactive hyperemia is due primarily to the accumulation of histamine or a histamine-like substance. The recent development of the antihistaminic drugs and the establishment of their histamine antagonizing property suggested a method whereby the validity of this concept might be tested in man. While this work was in progress a report of a similarly directed study in the cat appeared.¹⁰

Method. Six subjects were studied. These included 5 male patients hospitalized for reasons other than cardiovascular disease and one hypertensive female patient before and 24 days after combined nephrectomy and unilateral sympathectomy. Measurement of blood flow in the foot and distal portion of the leg was made by means of a venous occlusion volume recording plethysmograph according to principles previously described.¹¹ This en-

tailed placing the part of the limb to be tested within an airtight chamber. At the proximal margin of the plethysmograph a cuff 4 cm wide was placed around the leg and used to produce venous occlusion. A cuff 17 cm wide was placed about the thigh for the purpose of producing arterial occlusion. Both cuffs were attached to tanks in such a manner that by means of a system of valves any desired pressure could be rapidly obtained or released. The majority of studies were made after the subject had an average hospital breakfast, and they extended over 2 to 3 hours. Temperatures within the plethysmographic chamber were between 25.6° and 29.4° C. After at least 30 minutes of rest in the supine position determinations of resting flow were made as frequently as every 20 seconds. Reactive hyperemia was produced by the inflation and release of the arterial occlusion cuff. The cuff was rapidly inflated to approximately 220 mm Hg and the occlusion was maintained for periods of one-half to 10 minutes. Just prior to release the venous occlusion cuff was inflated and thus the initial inflow rate immediately following the release of arterial occlusion could be recorded with minimal distortion. The initial inflow rate is taken as representative of the degree of vasodilatation resulting from the occlusion. The subsidence of the vasodilatation was noted by repeating determinations of flow as often as every 15 seconds. At least one minute after subsidence of hyperemia the arterial occlusion was repeated, until a number of measurements of hyperemia were made. After completion of these control observations under normal conditions, "benadryl" (beta-dimethyl-aminoethyl-benzhydryl ether hydrochloride) 10 to 50 mg intravenously, or "pyribenzamine" (beta-dimethyl-aminoethyl - 2 - pyridyl-benzyl ammonium chloride) 50 mg orally, was administered. Observations were repeated at intervals for more than an hour.

The volume of the portion of the limb tested was measured by water displacement, and flows were expressed in terms of cc/min./100 cc limb. In 2 of the subjects intradermal injections of 0.1 cc containing 0.01 to 0.1 γ of histamine base were used to demonstrate

⁵ Barsoum, G. S., and Smirk, F. H., *Clinical Science*, 1936, **2**, 353.

⁶ Anrep, G. V., Barsoum, G. S., Salama, S., and Souidan, Z., *J. Physiol.*, 1944, **103**, 297.

⁷ Emmelin, N., Kahlson, G., and Wicksell, F., *Acta Physiol. Scand.*, 1941, **2**, 110.

⁸ Kwiatkowski, H., *J. Physiol.*, 1941, **100**, 147.

⁹ Hamilton, W. F., Chapter 33 in Howell's Textbook of Physiology, edited by J. F. Fulton. 15th Ed., 1946, Philadelphia, W. B. Saunders.

¹⁰ Emmelin, K., and Emmelin, N., *Acta Physiol. Scand.*, 1947, **14**, 16.

¹¹ Landowne, M., and Katz, L. N., *Am. Heart J.*, 1942, **23**, 644.

TABLE II.
Effect of Antihistaminic Drug Upon Whealing Produced by Intradermal Histamine.
(0.1 cc of a concentration of 1.0 γ per cc of histamine base was injected intradermally before and 17 min. after 30 mg of "Benadryl" intravenously.)

Min. after injection		Before drug	After drug
3	size of wheal, mm	25 \times 14	15 \times 12
	" " erythema, mm	30 \times 35	20 \times 20
13	wheal	22 \times 20	15 \times 14
	erythema	40 \times 45	33 \times 33
23	wheal	22 \times 20	15 \times 12
	erythema	47 \times 55 intense	33 \times 33 fading

flow, quantitation of either the total amount or of the duration of the increased circulation is not feasible under these conditions. No qualitative differences were observed in these aspects of reactive hyperemia after antihistaminic drug.

In comparing the period before administration of the antihistaminic drug with that afterward it will be noted that no significant effect of the drug upon blood flow is demonstrated. In some experiments there was a slight average increase in resting flow, and a comparable average increase in hyperemic flow. The air temperature within the plethysmographic chamber rose 0.5° to 2.5°C, an average increase of 1.1°C. The increase in local temperature, further relaxation of the subject, and any effects of specific dynamic action are factors which may account for a slight increase in flow during the latter part of an experiment.

The effectiveness of the antihistaminic agents used is indicated by the illustrative experience presented in Table II. The wheals and surrounding erythematous halo were smaller in size and persisted for a shorter period of time when histamine in graded dosage was injected after the administration of the drug.

Discussion. The data presented demonstrates the failure of antihistaminic drugs to reduce the vasodilatation of reactive hyperemia in the human limb. This confirms the work of Emmelin and Emmelin¹⁰ who were unable to show in the cat that antihistaminic drugs reduced the hyperemic volume increase following circulatory arrest.

In our studies an antihistaminic drug was carried to the tissues by means of the circulation. The concentration attained was sufficient to decrease the *local* intradermal whealing effect of 0.1 cc of concentration of 1.0 γ per cc of histamine base. Lewis considered³ that histamine introduced into the skin acted upon the walls of the minute vessels. Emmelin and Emmelin¹⁰ showed that the effect of intra-arterial histamine upon the minute vessels of the cat's hind limb could be reduced by an antihistaminic drug. Thus *exogenous* histamine, applied either from the outside or from within the lumen of the vessel, may have its effect upon the vessel wall reduced by the antihistaminic drug. The mechanism of the histamine blocking ability of these drugs is not known, but it has been suggested¹² that it is due to a competitive or combining property of the drug at the site of action of histamine rather than with histamine itself. This site of action would be at some portion of the cell surface or within the particular cells concerned in effecting the response. The inhibition of the action of *exogenous* histamine by an antihistaminic drug would indicate that the antihistaminic had reached this site of action. In this event, the effect of *endogenous* histamine, if produced outside or within the particular cells of the blood vessels which cause the vasodilatation of reactive hyperemia, should also be inhibited by circulating antihistaminic drugs.

The alternative possibility, that the effector

¹² Friedlaender, S., and Feinberg, S. M., *J. Allergy*, 1946, 17, 129.

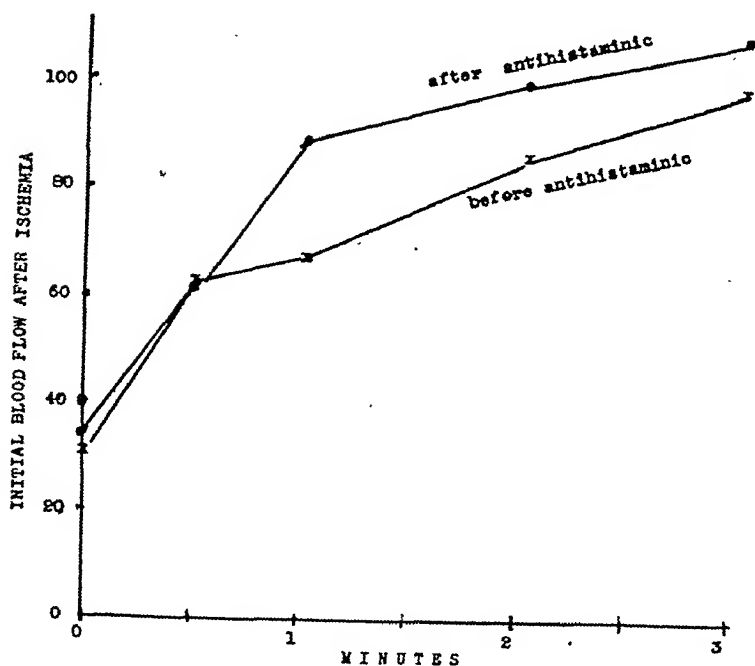


FIG. 1.

The effect of an antihistaminic drug upon blood flow at rest and during reactive hyperemia following arterial occlusion of $\frac{1}{2}$, 1, 2, and 3 minutes, respectively. Averaged data from five subjects. The "control" hyperemia after three minutes of ischemia has been arbitrarily given the value of 100.

the effectiveness of the antihistamine drug. This was done simultaneously with the blood flow studies.

Results. Average blood flows recorded before and after the administration of the antihistaminic drug during the resting state and at the onset of reactive hyperemia are summarized in Table I. Each value for resting flow represents the average of seven to 30 determinations, a total of 134 before and 99 after the drug. The average difference in average resting flows before and after administration of the drug is 0.08 cc, a decrease of 3%. The average of the per cent difference in each subject is plus 16%, due to low resting control flows in two cases.

Each value for hyperemia represents the flow after either one to 4 periods of occlusion of the duration indicated. Hyperemia was measured 39 times before and 36 times after the drug. Nineteen grouped comparisons were made. In only 4 of these was a decrease in flow noted after administration of the anti-

histaminic drug. In all other comparisons greater hyperemic flow was recorded after giving the drug than before. The average change in hyperemia for all groups was 0.65 cc/min. (range ± 2.20 cc/min.). The average change for each subject was 0.54 cc/min. (range -0.20 to + 1.43 cc/min.); and the average change after occlusions of like duration was 0.46 cc/min., or 5.5%. The average percentage change in each of these categories of comparison was between 10 and 15%.

In Fig. 1 are presented the averaged results in 5 experiments before and after the administration of the antihistaminic drug comparing flow at rest and during reactive hyperemia where arterial occlusions of one-half, one, 2 and 3 minutes respectively were used. The initial hyperemic flow after 3 minutes of occlusion before the drug was given is arbitrarily taken as a comparison standard of 100.

The duration of reactive hyperemia was brief, particularly after the shorter occlusions. Because of the normal fluctuations in resting

TABLE I.

The Nucleic Acid and Nitrogen Content of the Whole (W) and Residual (R) Chromosome Fractions of Normal and Leukemic Mouse Spleen.

	Exp. No.		Nitrogen—R/W, %	DNA, γ/γ N	PNA, γ/γ N
Normal	1	W		2.45	
		R	14	0.15	0.18
	2	R	—	0.13	0.19
		W		2.08	
	3	R	18	0.05	0.19
Leukemic	1	R	14	0.17	0.32
	3	W		2.77	
		R	23	1.21	0.36
	4	W		2.52	
		R	15	0.19	0.45

"residual chromosomes," which contain protein, pentose nucleic acid (PNA), and possibly a small amount of DNA. In this laboratory the whole and residual chromosomes of normal and leukemic mouse spleen have been isolated by the method of Mirsky and Ris.¹ The preparations have been examined microscopically and analyzed chemically for their DNA, PNA, and nitrogen contents.

Materials and methods. Mice of the Akm strain, about 3 months of age, were used. When injected with leukemic spleen minced in saline (strain 9421) they developed advanced leukemia in 8 or 9 days. The mice were sacrificed by spinal fracture, and the spleens removed immediately. The spleens of the normal controls averaged 180 mg in weight, while the leukemic spleens averaged 600 mg in weight. About 9 g of spleen were homogenized in 0.88 M sucrose, for cell fractionation studies which will be described elsewhere.² The nuclear fraction was resuspended in 0.15 M NaCl. The nuclei were broken in a refrigerated Waring mixer and the chromosomes isolated.¹ The progress of the isolation was followed microscopically by smears stained with Wright's stain.

Samples of the whole chromosomes were fixed and stained with Feulgen-light green and by other methods, and examined microscopically for traces of cytoplasm.¹ The nucleohistone was removed by extraction with M NaCl until the washings showed practically no absorption of light at 260 m μ . The

residual chromosomes were suspended in M NaCl. Nitrogen was determined by Nesslerization.³ The nucleic acids were extracted with hot trichloroacetic acid and analyzed for DNA by the diphenylamine test and for PNA by the orcinol test.⁴

Results. The whole chromosome fraction was fairly free of light green-staining material on microscopic examination. The residual chromosomes were badly clumped and could not be examined in detail.

Chemical analysis (Table I) showed that the DNA content of the leukemic chromosomes was the same as or slightly higher than that of the normal chromosomes. Both sets of values correspond to a DNA content of about 37% of the total chromosome, which is similar to the values found for "chromatin threads" from leukemic mouse spleen and rat leukemic tumors⁵ and for whole chromosomes from calf thymus.¹

The PNA content of the whole chromosome fraction, as determined by the orcinol reaction in the presence of a large excess of DNA, is only an approximate value. Only traces were found in the normal chromosome fraction, and slightly more (0.1 γ per γ of N) in that from leukemic spleen.

The residual chromosome fraction from

³ Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric techniques and related methods for the study of tissue metabolism*, Minneapolis, 1945.

⁴ Schneider, W. C., *J. Biol. Chem.*, 1945, **161**, 293; 1946, **164**, 747.

⁵ Claude, A., and Potter, J. S., *J. Exp. Med.*, 1943, **77**, 345.

² Petermann, M. L., Alfin-Slater, R. B., and Larack, A. M., *Cancer*, in press.

¹ Through the courtesy of Dr. John J. Bieseke.

cell is not penetrated by the antihistaminic drug, requires that the site of action of histamine be at a surface to which *exogenous* histamine is carried, by the circulation, by penetration through cells, or by passage between cells. In this circumstance it is conceivable that *endogenous* histamine may, by arising within the cell, reach and act upon the surface in a manner not open to inhibition by the agent used. This is unlikely. "Benadryl" is a small, ionizable molecule which readily passes from the circulating blood to the extracellular spaces. There is no evidence to indicate that it is unable to traverse the cellular structure of the vessel wall in its passage. Clinical studies¹³ have shown that those allergic manifestations held to be due to an increase in *endogenous* histamine activity are reduced by antihistaminic drugs. This requires that the drug reach the site of action of the *endogenous* histamine liberated in these disorders.

The term "antihistaminic" indicates that these drugs possess the ability to inhibit the biological effects of histamine. It would be expected that a "histamine-like" substance "with the biological properties of histamine" would be similarly inhibited. This cannot be determined, of course, until such substance is actually demonstrated.

Thus the failure of antihistaminic drugs to reduce the vasodilatation of reactive hyper-

emia in man can be considered to mitigate strongly against the generally accepted concept that histamine or a histamine-like substance is the primary factor in the vasodilatation of reactive hyperemia.

The unmodified persistence of the capacity for reactive hyperemia after sympathetic denervation indicates, as Lewis pointed out,³ that this phenomenon is not dependent upon sympathetic integrity. The antihistaminic drug failed to modify reactive hyperemia in the sympathectomized limb. This demonstrates that the drug does not normally tend to evoke a compensatory reduction in sympathetic activity which could mask an effect on reactive hyperemia.

Summary and Conclusions. 1. In 6 subjects reactive hyperemia was produced in the legs by release of arterial occlusion maintained for from one-half to 10 minutes. Blood flow in the foot and distal portion of the leg was measured with a venous occlusion plethysmograph. The initial inflow rate immediately following release of arterial occlusion is taken as representative of the degree of vasodilatation resulting from the occlusion.

2. After administration of effective amounts of antihistaminic drugs the reactive hyperemia was not diminished.

3. The mechanism of reactive hyperemia has not been shown to be due to release of histamine or "H-substance." It is as yet unexplained.

¹³ Feinberg, S. M., *J. A. M. A.*, 1946, **132**, 702.

16782 P

Nucleic Acid Content of Chromosomes of Normal and Leukemic Mouse Spleen.*

MARY L. PETERMANN AND ETHEL J. MASON. (Introduced by C. P. Rhoads.)

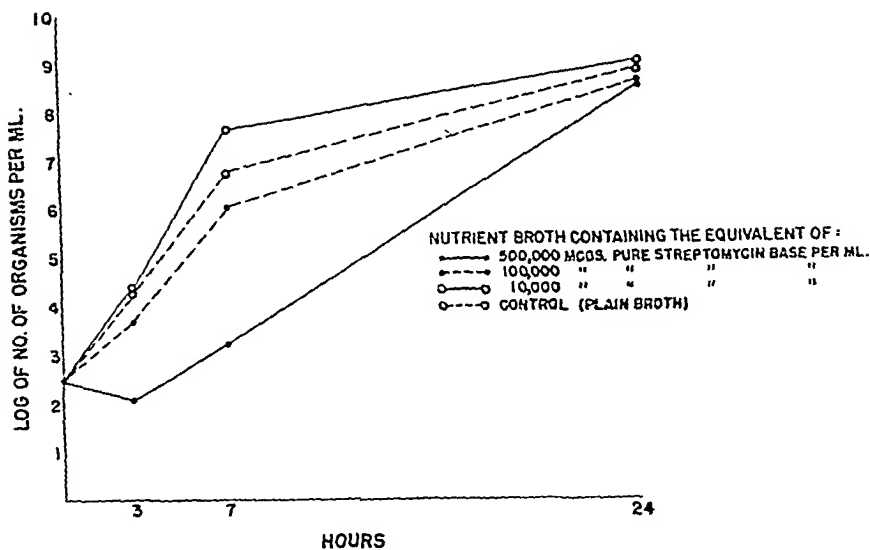
From the Sloan-Kettering Institute for Cancer Research, New York City.

It has recently been shown by Mirsky and

Ris¹ that chromosomes may be isolated from the resting nucleus. These isolated chromosomes are made up chiefly of desoxyribose nucleic acid (DNA), histone, and the

* The expenses of this investigation were defrayed by grants from the Office of Naval Research, the National Cancer Institute of the United States Public Health Service, the Finney-Howell Foundation, and the James Foundation of New York, Inc.

¹ Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, 1947, **31**, 1, 7.



GRAPH 1.

Effect of streptomycin on the growth of a streptomycin-resistant gram negative rod (strain SMR).

Isolation. An aqueous solution of a highly purified form of streptomycin sulfate, prepared through an intermediate crystalline stage, in a concentration equivalent to 375,000 μg of pure streptomycin base per ml, was allowed to stand at room temperature for a period of several days. Subcultures were subsequently made from the solution (1) by streaking 0.05 ml of the undiluted solution onto beef infusion agar and onto Sabaraud's agar, and (2) by inoculating 0.1 ml of the undiluted solution and a 10^{-5} dilution of the solution into beef infusion broth and into fluid thioglycollate medium. Incubation was carried out at 5°C , 22°C , and 37°C . Luxuriant growth appeared in all cultures incubated at 22°C and at 37°C . No growth occurred at 5°C .

Cultural characteristics. Morphologically the organism is a short plump gram negative rod with rounded ends and varying slightly in size. At times the organism may appear almost coccoid in form. It is nonmotile and produces no spores.

The organism grows readily in diffuse form under aerobic or facultative anaerobic conditions in liquid media.* Growth occurs at pH ranging from 6.0 to 7.8 and at room temperature (22°C) and 37°C ; no growth

occurs at 5°C .

On plain or blood agar the organism grows in the form of a smooth opaque grayish-white colony, approximately 3-4 mm in size. It is nonhemolytic. On glucose or Sabaraud's agar, it grows in the form of a confluent viscous or mucoid slime.

Growth occurs rapidly in the presence of glucose, maltose, sucrose, levulose, arabinose, and d-galactose with the formation of acid and gas. In the presence of xylose, acid without gas is formed, while in the presence of lactose (0.1%), growth is slow and no acid is produced.

Growth occurs readily on sodium citrate agar. The organism is capable of reducing nitrates to nitrites. Acetyl methyl carbinol is not formed. In Voges-Proskauer-Methyl-Red broth, a powerful reducing substance is produced. In distilled water† and in distilled

* It is of interest to note that this organism is incapable of producing detectable amounts of penicillinase. It is sensitive to 80 units of penicillin per ml and to the equivalent of 40 μg of polymyxin B hydrochloride per ml.

† Baxter sterile distilled water was used in all experiments throughout this study. Analyses indicated a content of less than 10 p.p.m. total solids.

both normal and leukemic spleens contained about 17% of the nitrogen of the whole chromosome fraction (Table I). The PNA contents, on the other hand, were strikingly different. Whereas the residual chromosome fraction from normal mouse spleen contained only 0.18-0.19 γ of PNA per γ of nitrogen, that from leukemic spleen contained 0.32 to 0.45 γ of PNA per γ of nitrogen, an increase of nearly 100%.

Discussion. It is of great importance to determine whether the excess PNA found in the residual chromosome fraction of leukemic spleen is an integral part of the chromosome structure or is contained in some other constituent of the preparation. The cytoplasm of the leukemic spleen cells contained large amounts of PNA.² That any significant amount of cytoplasm was left in these preparations seems unlikely, however, since careful microscopic observation failed to reveal it. The whole "nuclear fraction" of the leukemic spleens, however, was also extremely rich in

PNA.² This is in agreement with the findings of Thorell,⁶ who has shown, by ultraviolet spectrophotography, that the nucleus of the leukemic white cell contains 2 to 4 nucleoli rich in PNA, which are absent from the normal mature white cell. Since nucleoli sometimes remain attached to isolated chromosomes,¹ it is probable that some of the excess PNA found in the leukemic residual chromosome preparations is located in associated nucleoli; how much cannot be decided until preparations are obtained which do not clump so badly, and can be examined microscopically.

Summary. The residual chromosome fraction isolated from leukemic mouse spleen contained from 0.32 to 0.45 γ of pentose nucleic acid per γ of nitrogen, while that from normal spleen contained 0.18-0.19 γ per γ of nitrogen.

⁶ Thorell, B., Studies on the formation of cellular substances during blood cell production. *Lundon*, 1947.

16783

Isolation of a Streptomycin-Resistant Organism Capable of Utilizing Streptomycin for Growth.

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Since the introduction of streptomycin in 1944, the appearance of streptomycin-resistant variants in otherwise sensitive strains of microorganisms has been observed repeatedly. Miller and Bohnhoff¹ reported the isolation of variants of meningococci not only resistant to streptomycin but capable of growing only in its presence. These organisms were dependent upon streptomycin for multiplication both *in vitro* and *in vivo*. Paine and Finland,² described dependent variants of other bacterial species including *Staphylococcus aureus*, *E.*

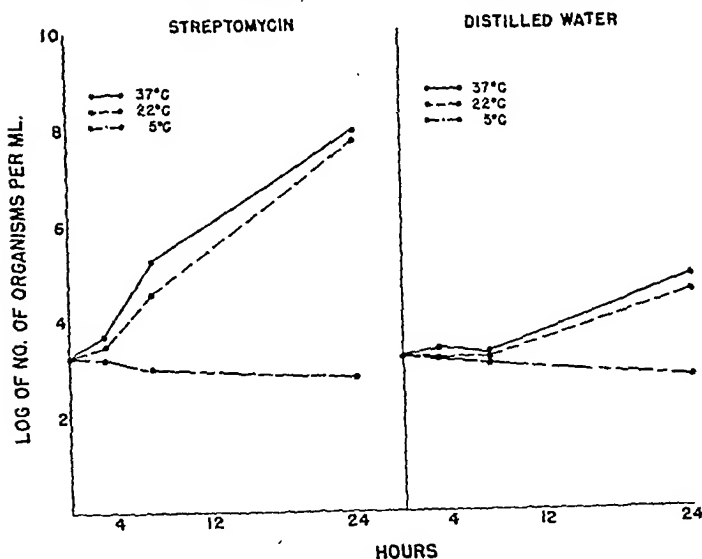
coli, *Ps. aeruginosa*, and *P. morgani*. More recently, 13 streptomycin-dependent strains of *M. tuberculosis* have been isolated in our own laboratory³ from mice infected with the H₃₇Rv strain and subsequently treated with streptomycin for a period of 31 days. The possibility that these organisms are capable of utilizing the nitrogen of streptomycin for growth has been suggested.

In the present communication, the isolation of a streptomycin-resistant organism capable of growing both in streptomycin as the sole source of nutrient and in broth containing no streptomycin is discussed.

¹ Miller, C. P., and Bohnhoff, M., *Science*, 1947, 105, 620.

² Paine, T. F., and Finland, M., *Science*, 1948, 107, 143.

³ Lenert, T. F., and Hobby, G. L., *Am. Rev. Tuberculosis*, 1949, in press.



GRAPH 2.

The rate of growth of a washed suspension of a streptomycin-resistant gram negative rod in presence of streptomycin as source of nutrient.

When heat-killed suspensions of the organisms, grown on broth or agar, were injected intravenously into each of 3 rabbits, all of the animals showed marked pyrogenic responses consisting of temperature rises ranging from 1.2 to 2.2°C. These animals appeared ill soon after injection and 7 of the 9 died within 24 hours.

Seven-tenths of a ml of each of these suspensions, when administered intravenously to mice (Swiss strain), produced no reaction. It seems possible, therefore, that death of the rabbits receiving this material may have been due primarily to pyrogenic reactions rather than to the presence of other toxic substances.

All animals receiving the aqueous suspension of standard pyrogen prepared from *Ps. pyocyaneus*[†] survived with no untoward reaction other than rise in temperature.

Discussion. The isolation of an organism which is not only resistant to the antibacterial

action of streptomycin but capable of multiplying in the presence of streptomycin as the sole source of nutrient has been described. Recently a second organism has been isolated which is also resistant to streptomycin and capable of growing in high concentrations of the drug. This organism is a slender gram-negative rod which grows on beef infusion agar in the form of an opaque creamy yellow colony, 2-3 mm in diameter. On blood agar it produces a grayish-red pigment after 1 to 2 weeks at room temperature. Sugars are not fermented readily.

The fact that such organisms may exist is significant. It is now apparent that microorganisms may be divided into 4 general categories, in relation to streptomycin: (1) the streptomycin-sensitive cells, including those that are capable of growing in culture media without streptomycin but are inhibited by the presence of streptomycin, (2) the streptomycin-resistant cells, which are capable of growing either in culture media alone or in media containing streptomycin, (3) streptomycin-resistant cells, which are capable of multiplying in culture media, in media containing streptomycin, or in aqueous solutions of streptomycin, and (4) streptomycin-dependent

[†] Prepared from a culture of *Ps. pyocyaneus* according to the method of Welch, Calvery, McClosky, and Preece⁴ and subsequently purified further by removal of protein.

⁴ Welch, H., Calvery, H. O., McClosky, W. T., and Preece, C. W., *J. Am. Pharm. Assn.*, 1943, 32, 65.

water† containing 3% methyl or ethyl alcohol, growth occurs rapidly within 24 hours.

Although morphologically suggestive of the *Pseudomonas* group of organisms, the data above are not sufficient to place this organism within this species.

Streptomycin Resistance. In view of the fact that this organism was originally isolated from a solution containing a high concentration of streptomycin, it is apparent that it is highly resistant to this antibacterial agent.

Graph 1 shows the rate of growth of the organism in broth containing varying concentrations of highly purified streptomycin. Highly purified streptomycin sulfate was diluted to 10,000, 100,000, and 200,000 μg of pure streptomycin base per ml. Plain broth was used as control. An 18-hour broth culture of the streptomycin-resistant organism was diluted in broth to a dilution of 10^{-5} , and 0.5 ml of this was inoculated into tubes containing 4.0 ml of each of the above solutions of streptomycin in broth. Incubation was carried out at 37°C . The number of organisms per cc was determined by colony counts at 0, 3, 7, 24 hours. No inhibition of growth was observed in the presence of streptomycin in concentrations equivalent to 10,000 or 100,000 μg of pure base. A concentration of streptomycin equivalent to 500,000 units of pure base produced a temporary lag in the growth of the organism, lasting for a period of 3 to 7 hours only. After 24 hours incubation, the number of organisms per ml was as great in the presence of the equivalent of 500,000 μg of pure streptomycin base per ml as in broth containing no streptomycin.

Utilization of streptomycin for growth. The fact that this organism was originally isolated from a solution of streptomycin in water suggested that the organism must be capable of utilizing streptomycin for growth.

Highly purified streptomycin sulfate was diluted in sterile distilled water to a concentration equivalent to 333,000 μg of pure streptomycin base per cc. Four cc of this solution was pipetted into each of 3 tubes. As control, 4 ml of sterile distilled water was pipetted into each of 3 additional tubes. An 18-hour broth culture of the streptomycin-re-

sistant strain was centrifuged and the sedimented organisms washed twice with sterile distilled water to remove traces of broth. The organisms were then resuspended in a volume of sterile distilled water equal to that of the original broth culture. The aqueous suspension of organisms was then diluted to 10^{-1} , and 0.1 ml of this dilution was inoculated into each of the 3 tubes of streptomycin solution and into each of the 3 tubes of sterile distilled water. Incubation was carried out at 37°C .

Two similar sets of tubes were prepared and incubated at 5°C and at 22°C , respectively.

The results are shown in Graph 2. No multiplication occurred at 5°C either in the aqueous solution of streptomycin or in distilled water. At both 22°C and 37°C however, heavy growth occurred in the aqueous solution of streptomycin. Whereas the rate of growth was somewhat slower than had previously been observed when the organism was grown in a broth solution of streptomycin (Graph 1), the number of organisms per ml after 24 hours incubation was as great in the aqueous solution of streptomycin as it previously had been in the broth solution of streptomycin. At 22°C and at 37°C , slight multiplication took place in the distilled water. The amount of growth occurring in the water alone was, however, far less than in the aqueous solution of streptomycin, and it is apparent, therefore, that this organism is capable of utilizing streptomycin for growth.

Pathogenicity. Mice of the Rockland regular strain inoculated intraperitoneally with an undiluted 4-hour plain broth culture died within less than 72 hours; mice similarly inoculated with dilutions of 10^{-1} – 10^{-7} all survived, indicating that the organism possesses little virulence for mice.

It has been observed by Miller and Bohnhoff that streptomycin-dependent strains of meningococci are virulent for mice only if the animals are treated simultaneously with streptomycin. The virulence of this strain, therefore, was tested in mice receiving 4 mg of streptomycin daily by the subcutaneous route. Again only those animals receiving undiluted culture failed to survive.

cells, which multiply only in the presence of streptomycin. The frequency with which streptomycin-resistant variants and organisms capable of utilizing streptomycin for growth have been described has limited its usefulness

as a chemotherapeutic agent.

Conclusions. The isolation and cultural characteristics of a streptomycin-resistant organism capable of multiplying in aqueous solutions of streptomycin has been described.

16784 ✓

Biological Studies with Arsenic⁷⁶. II. Excretion and Tissue Localization.

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AND AUSTIN M. BRUES.

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Arsenic⁷⁶ is characterized by a β radiation maximum of 3.04 MEV, a γ radiation maximum of 2.15 MEV, and a 26.8 hour half-life. The high energy beta and gamma emission simplify counting but necessitate shielding to protect personnel. The short half-life makes tracer experiments of long duration impossible; but the rapid decay is useful in therapeutic applications, as radiation dosage is easily controlled.

In order to calculate radiation dosage from any radioactive material to a particular tissue, it is essential to know not only the physical characteristics of the isotope employed, but also the concentration of the material in the tissue under consideration. For this reason, as well as for the more theoretical purposes discussed in an earlier paper,¹ we began a series of studies on the fate of radio-arsenic in laboratory animals, and, later, in man. Several phases of this research still are far from complete; however, because of the therapeutic potentialities of arsenic⁷⁶,² and because radioisotopes of arsenic are now offered for general distribution,³ we feel that our studies on dis-

tribution and excretion may be of some interest and value to other experimenters.

Early work with radio-arsenic. Radioactive arsenic has been previously reported upon by Lawton and co-workers,⁴ who administered 16-day arsenic (As^{74}) to a group of 6 cotton rats infected with *Litomosoides carinii*, sacrificing their animals 24 hours after intraperitoneal injection; by DuPont *et al.*,⁵ who injected radio-arsenic intravenously in 37 rabbits; and by Hunter and co-workers,⁶ who studied distribution in tissues, and in various chemical fractions of tissue, of radio-arsenic administered subcutaneously to rats, rabbits, guinea pigs, higher apes, and man. All of these experiments showed a remarkable degree of individual variation among animals of the same species receiving apparently identical treatment.

In general, all animals in all experiments exhibited greatest arsenic concentration in liver, kidney, spleen, and lung. Low arsenic uptake by the Brown-Pearce rabbit tumor was found by the DuPont group, who also reported no change in the tissue distribution pattern of tumor-bearing animals. Hunter *et al.* noted a remarkable difference between

¹ Straube, R. L., Neal, W. B., Jr., Kelly, T., and Ducoff, H. S., Part I. *Proc. Soc. Exp. Biol. and Med.*, in press.

² Neal, W. B., Jr., Jacobson, L. O., Brues, A. M., Ducoff, H. S., Straube, R. L., and Kelly, T., *Am. Assn. for Cancer Research*, March 13, 1948, Atlantic City, N. J.

³ U. S. Atomic Energy Commission. *Radioisotopes—Catalogue and Price List*, No. 2, September, 1947.

⁴ Lawton, A. H., Ness, A. T., Brady, F. V., and Cowie, D. B., *Science*, 1945, **102**, 120.

⁵ DuPont, O., Ariel, I., and Warren, S. L., *Am. J. Syph. Gon. and Ven. Dis.*, 1942, **20**, 96.

⁶ Hunter, F. T., Kip, A. F., and Irvine, J. W., Jr., *J. Pharm. and Exp. Therap.*, 1942, **70**, 207; Lowry, O. H., Hunter, F. T., Kip, A. F., and Irvine, J. W., Jr., *ibid.*, 1942, **70**, 221.

TABLE III.
Tissue Distribution, Rats ($\mu\text{mc/g}$).

Sacrificed at Dose Organ	6 hr		24 hr		48 hr		96 hr	
	230	295	336	324	174	313	350	255
Blood	1705	2360	2780	2820	2600	2160	1390	1990
Spleen	603	662	990	1420	448	900	540	
Heart	538	628	700	840	239	266	328	365
Lung	442	420	542	598	512	356	610	460
Kidney	396	345	382	347	184	255	157	186
Adrenal	283		255	323	298	213		300
Thymus	200	407	142	388	186	304	630	254
Liver	174		365		148	159	158	104
Testis	43	65	74	80	22	41		28
Muscle	58	50	41	42	11	23	22	12
Skin and fur	47		64	70			38	78

TABLE IV.
Distribution of Arsenic in Tissues of Rabbits ($\mu\text{mc/g}$).

Sacrifice time (hr after inj.)	6	24	48	96
Sex	♀	♂	♂	♀
Dose	72	82	76	70
Blood	23	9	3	
Spleen	63	22	19	
Heart	46	13	3	3
Lung	104	72		
Kidney	168	46	15	
Liver	415	62		24
Muscle	36	27	10	5
Femoral marrow		186	39	35
Brain	12	10	2	

blood is very striking. The fairly high arsenic content of spleen and lung, the large individual variation, and the lack of any great tendency for a decline in the arsenic content of most tissues, even after 48 hours, are also noteworthy. Inspection suggests that the high spleen values may be explained by the blood contained therein.

Rabbits: Table IV presents the data on tissue localization in the 4 rabbits used in the excretion studies (above). This pattern is in distinct contrast to that found with rats, in that concentrations of arsenic in blood after any time interval studied are never as great as in most of the solid tissues, and all tissues show a distinct reduction in arsenic content as time progresses. Liver, kidney, and lung contain the highest concentrations of arsenic.

Mice: Tissue localization of arsenic was studied in several series of mice, some bearing transplantable tumors. Tumors used were:

1. The Jackson-Brues embryoma,⁸ which is grown in C₃H mice, develops slowly, and is quite variable as to rate of growth, percentage of "takes," and tissue organization; and

2. A lymphoma[†] which is grown in A mice, kills the host 4 to 6 weeks after transplantation, has 100% "takes," and is quite homogeneous.

Arsenic concentration was determined, as a rule, only in kidney, liver, lung, spleen, muscle, and tumor, when present. In all experiments, mice were injected intraperitoneally.

In the first study on mice 19 males of the A strain, 11 bearing lymphomas that had been transplanted 3 weeks previously, received 0.8 μc (in 0.25 ml) each. Table V records the results obtained in this experiment. Although this is a highly inbred strain, and although all efforts were made to treat the mice identically, the degree of individual variation within any

* Obtained from Dr. J. C. Aub of Harvard University.

⁸ Jackson, E. B., and Brues, A. M., *Cancer Research*, 1941, 1, 494.

[†] Kindly supplied by Dr. Egon Lorenz.

TABLE II.
Excretion in Man.

Patient Diagnosis Dose	G. A. Hodgkin's Disease 3.0 millicuries			G. R. Lymphatic leukemia 2.7 millicuries		
	Urine (μ c)	Feces (μ c)	% of dose excreted	Urine (μ c)	Feces (μ c)	% of dose excreted
1st 24 hr	498	2.0	16.7	116		4.3
2nd " "	548	3.7	18.5	574		21.5
3rd " "	288		9.6	194	5.6	7.4
4th " "	149	14.6	5.4	234	6.8	8.6
5th " "	260	3.6	8.8	296		11.0
6th " "	124		4.1	183		6.8
7th " "	104	5.2	3.7			

Figures on activity in all samples of tissues and of excreta are corrected for decay to the time of injection. Thus, activity is always proportional to the true amount of the isotope present for animals treated with the same injection solution, and animals of different treatment groups may be compared on the basis of "per cent of injected dose."

Excretion studies. Rats: Five male Sprague-Dawley rats, injected in the tail vein with 47 μ c (0.2 cc), were immediately placed in metabolism cages, and excreta were collected at 24 hour intervals. Activity in the fecal samples was too low to count; Table I shows the data on the urine samples.

Rabbits: Four stock rabbits each received 235 μ c (1 cc) via ear vein. Excreta were collected at intervals of 24 hours, except in the case of rabbit No. 39, which was sacrificed 6 hours after injection. Data on rabbit excreta are included in Table I.

Man: Table II summarizes the data on arsenic excretion in 2 representative patients injected intravenously.

Mice: No exact measurements were made on excretion rates in mice, but surveys with a portable meter on mouse cages and mice injected intraperitoneally indicated that some 75% of the injected dose is excreted within the first 24 hours.

It can be seen from the tables, and from Fig. 1, which summarizes the data, that arsenic excretion takes place far more slowly in rats (<10% the first 48 hours) than in

rabbits (70%) or man (30-45%). More significant, probably, is the rapidity with which the arsenic⁷⁶ content of rats comes to equilibrium, as illustrated by the leveling off of the excretion curve.

In all species studied, including the rat, the feces account for less than 10% of the total arsenic excreted.

Tissue distribution patterns. Rats: Eight Sprague-Dawley males injected intravenously with 47 μ c each were sacrificed in pairs at 6, 24, 48, and 96 hours. The results are shown in Table III.

The high concentration of arsenic in the

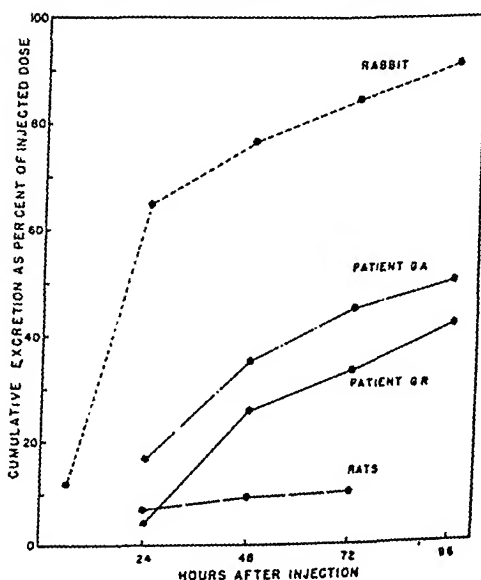


Fig. 1.

Excretion of Arsenic⁷⁶. Cumulative excretion from the time of injection is expressed as percentage of the administered dose.

TABLE VI.
Kidney-Liver-Spleen Ratios in Mice.

Strain	Hr after inj.	Proportion (% basis)					
		Non-tumorous			Tumor-bearing		
		Kidney	Liver	Spleen	Kidney	Liver	Spleen
A	6	46	31	23	39	34	27
		45	32	24	38	35	27
A	12	29	31	40	31	41	29
		28	32	40	38	36	26
					37	40	23
C ₃ H	12	22	42	36	41	35	24
		21	47	32	34	42	24
		26	47	27			
		32	36	33			
		21	38	41			
		24	44	32			
		31	43	27			
		27	40	33			
C ₃ H	24	21	34	46	49	36	15
		20	40	41	18	45	36
		25	34	40			
		17	26	57			

TABLE VII.
Tissue Distribution in Man (Patient H. N.).
(20 hours after injection.)

Tissue	m μ e/g
Liver	46.4
Kidney	29.5
Spleen	16.1
Parotid tumor	15.6
Heart	14.6
Jejunum	14.3
Vertebral marrow	14.2
Mesenteric lymph node	12.8
Stomach	11.7
Pancreas	11.6
Muscle (quadriceps)	11.4
Pleum	11.1
Lung	10.8
Femoral marrow	10.8
Adrenal	8.5
Ovary	8.3
Thyroid	7.6
Skin	6.7
Brain	2.5
Femoral cortical bone	2.4

Man: A moribund 65-year-old female with carcinoma of the parotid was given 500 microcuries of As⁷⁶ (=4 mg arsenic) 20 hours before death. The distribution of activity in the tissues at time of death is shown in Table VII.

Arsenic levels in the blood. At the time of sacrifice of rabbits and of rats for distribution studies, specimens of blood were usually taken. Blood samples have also been obtained

from patients, and a series of samples was drawn from one chicken. The results of these determinations are plotted semi-logarithmically as a function of time after injection in Fig. 2. The great degree of arsenic retention in rat blood is illustrated both by the high level at any particular time, and by the low negative slope of the curve.

Discussion. The change in distribution of arsenic in non-tumorous organs of tumor-bearing mice appears to be an example of a systemic effect wrought by a (histologically) localized phenomenon. Arsenic is largely bound to protein,⁹ and, though to a lesser extent, to the -SH groups in cystine, glutathione, etc.⁹ The proportion of arsenic retained in a particular organ can presumably be altered by

- a change in the concentration of the arsenic binding constituents.
- an alteration in the arsenic-combining capacity of some chemical substance, or
- a combination of a and b.

Although the presence of tumor clearly alters the distribution of arsenic, the direction of the change in a particular organ (as, say,

⁹ Voegtlin, C., Dyer, H. A., and Leonard, C. S., *Public Health Reports*, 1923, 38, 1882.

TABLE V.
 Distribution of Arsenic in Tissues of "A" Mice.

Sacrificed	Tumor, + or 0	Dose, m μ c/g	Concentration m μ c As/g tissue				
			Kidney	Liver	Spleen	Tumor	Muscle
At 6 hrs	0	348	389	263	193		343
	0	334	286	202	154		85
	+	297	249	215	172	109	96
	+	276		218		144	188
	+	334	175	157	120	86	91
" 12 "	0	308	65	74	104		85
	0	320	63	70	85		82
	+	286	115	106	76	63	51
	+	268	87	92	54	33	48
	+	297	62	83	61	31	45
" 24 "	0	297	25*	30	23*		—
	0	320	31	28	—		—
	+	297	23*	25	23*	12	23*
	+	268	25*	37	—	11	—
" 48 "	0	348	—	10	—		—
	0	334	26*	14	—		—
	+	334	—	21	41	23	—
	+	334	—	20	—	7.6	—
	+	348	—	27	32*	8.8	—

— = Gave count of less than $1\frac{1}{2}$ times background.

* = Based on count between $1\frac{1}{2}$ and twice background.

Figures on activity and concentration have been corrected for decay to the time of injection.

1 m μ c = 0.003 μ g.

sacrifice group is extremely high.

It was noted, however, that the ratio of arsenic concentrations in kidney, liver, and spleen among non-tumorous mice of the early sacrifice groups appeared to be fairly constant. Accordingly, the per cent ratios of kidney, liver, and spleen concentrations were calculated as shown below:

Sample Calculation.			
Kidney-liver-spleen ratio, first animal in Table V.			
Organ	Arsenic in 1 g tissue	Absolute ratio	% ratio
Kidney	389 m μ c	339	389
		193	845 = 46%
Liver	263 m μ c	263	263
		193	845 = 31%
Spleen	193 m μ c	193	193
		193	845 = 23%
Sum	845	2.0:1.4:1.0	46-31-23

The calculated percentage ratios for these mice, and for a group of female C₃H mice, some bearing transplanted embryomas, which were injected intraperitoneally with 6.7 μ c,

are shown in Table VI. It is readily seen that though there is greater variation in the kidney-liver-spleen (KLS) ratios for this group of C₃H mice, the variation both from tumorless animals and from each other among the embryoma-bearers is quite striking.

The existence of a reproducible ratio of concentration between various tissues of normal mice, despite the remarkably wide range of variation in absolute values, may be explained, at least partially, on the following basis: The rapid rate of arsenic excretion by rabbits, mice, and man has been demonstrated. Any variation in excretion rate will therefore be greatly exaggerated unless concentration is expressed as percentage of retained, rather than total dose; e.g., a variation of 10% in the quantity excreted is equivalent to a variation of some 40% in the dose retained.

It should be noted, however, that rats, with a very low rate of excretion, show as high a degree of variation in concentration patterns as any other animal studied. This may be partly accounted for by variability in blood content of organs.

Anomalous Effects of Zinc-Insulin Upon *Trypanosoma hippicum*.*STEWART C. HARVEY,^{†‡} (Introduced by E. L. Smith.)

From the Department of Pharmacology, University of Chicago.

During the course of investigations into the nature of carbohydrate metabolism in *T. hippicum* some very interesting observations were made on the effect of zinc-insulin upon the respiration of the parasites.

Insulin has been reported to cause augmentation of the oxygen assimilation of pigeon breast muscle under certain conditions,¹⁻³ and a similar augmentation has been reported for mammalian tissues.⁴ The increase in oxygen utilization is thought to be concerned with pyruvate oxidation.⁵ *T. hippicum* responds to zinc-insulin with sizeable increases in oxygen uptake and pyruvate production, but glucose utilization is spared. Zinc-insulin inhibits the anaerobic production of pyruvate from glucose.

Experimental. Whole trypanosomes were suspended in a medium of Krebs Ringer-phosphate⁶ with 0.025 M bicarbonate added. Glucose was used as a substrate. Measurements of gas exchange were made manometrically at 38°C with a Barcroft-Warburg constant volume respirometer using the methods of Dixon.⁷ Glucose was analysed by the method of

INSULIN ON OXYGEN-UPTAKE

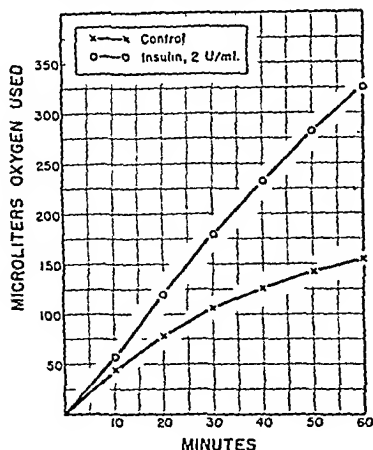


FIG. 1.

The augmenting action of zinc-insulin on the time variation of oxygen uptake. Glucose concentration, 0.01 M.

Nelson,⁸ and pyruvate was determined according to Friedemann and Haugen.⁹

The effect of zinc-insulin (Lilly) upon time variation of oxygen-uptake of *T. hippicum* is shown in Fig. 1. The response shown may be elicited when the insulin concentration is as low as 0.1 unit per ml of medium, but below that level the effect becomes insignificant. Under conditions of optimal glucose concentration (0.01 M) the trypanosomes utilize about 100% more oxygen during the first hour when insulin is present than when it is withheld. The augmentation of the initial rate, however, only amounts to about 20%. Insulin also has a marked effect upon the oxygen uptake-glucose concentration relationship. The slope of the ascending limb of the curve is greatly increased, and the plateau level of the curve is elevated 60-100%, as seen from the example given in Fig. 2.

* This investigation was supported by a grant-in-aid from the U. S. Public Health Service.

† Roche Anniversary Foundation Fellow in Pharmacology, 1947-1948.

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¹ Krebs, H. A., and Eggleston, L. U., *Biochem. J.*, 1938, **32**, 913.

² Shorr, E., and Barker, S. B., *Biochem. J.*, 1939, **33**, 1798.

³ Stadie, W. C., Zapp, J. A., and Lukens, F. D. W., *J. Biol. Chem.*, 1940, **132**, 411.

⁴ Stare, F. J., and Baumann, C. A., *J. Biol. Chem.*, 1940, **133**, 453.

⁵ Rice, L., and Evans, E. A., Jr., *Science*, 1943, **97**, 470.

⁶ Krebs, H. A., and Henseleit, K., *Z. Physiol. Chem.*, 1932, **210**, 33.

⁷ Dixon, M., *Manometric Methods*, Ed. 2, Cambridge, 1943.

⁸ Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

⁹ Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, 1943, **147**, 415.

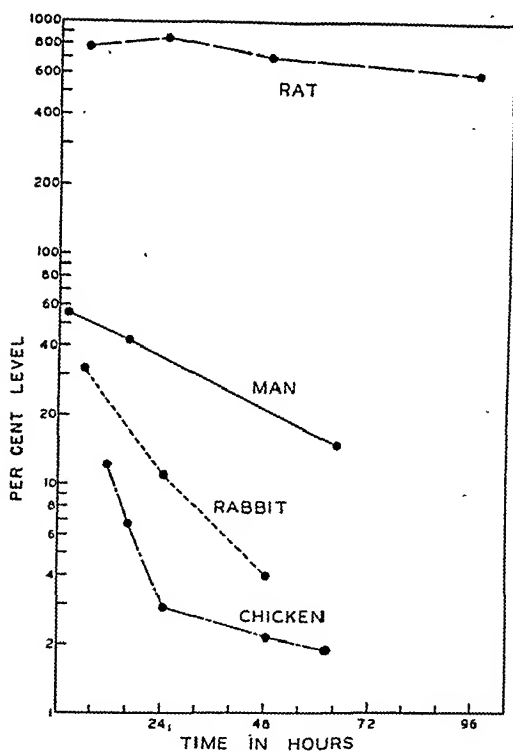


FIG. 2.

Arsenic⁷⁶ levels in whole blood. Concentration of arsenic⁷⁶ per gram of blood at a particular time is expressed as percentage of the administered dose per gram of body weight.

liver) is unpredictable.

Several workers have considered the effects of tumors on chemical composition of uninvolved organs, and the question has been reviewed in part by Toennies.¹⁰ Glutathione and cystine appear to have been especially altered in unaffected organs of tumorous animals, as reported by Voegtlin and Thompson, by Woodward, and by Schenk.¹¹ It should also be noted that changes in the -SH levels of blood plasma are found in the presence of

a great variety of tumors, and form the basis for several attempts at formulating sero-diagnostic tests for cancer.¹²

We have as yet been unable to find any unique physiological or chemical property of rat's blood which might explain the high degree of arsenic retention. The deceleration of excretion rate beyond the first day makes it appear that retention in the rat is not primarily due to inability to excrete arsenic; the high blood concentration with preference for erythrocytes⁶ suggests that the red cell of the rat may contain a system binding arsenic.

Summary. 1. Arsenic excretion was studied in man, rats, and rabbits. Less than 10% of the excreted arsenic is found in feces in any of these species; rats have by far the slowest rate of excretion.

2. Data are given for arsenic distribution in various organs in man, the rat, the rabbit, and 2 strains of mouse.

3. The degree of individual variation within each species was very great; in contrast to man and to other animals studied, the rat retains most of the injected dose in the blood for a considerable length of time.

4. The ratio of arsenic concentration in kidney, liver, and spleen of healthy inbred mice was found to be fairly constant for a given time after administration, and this ratio is suggested as a criterion for effects of various types of treatment.

5. Using this ratio as criterion, it was found that arsenic distribution is altered by the presence of transplanted tumors.

6. Factors changing arsenic distribution are discussed in relation to effects on levels of sulfhydryl-containing substances.

Acknowledgment is here made of the valuable assistance of Dr. Henry Hopple, Vladimir Clemens and Sarmukh Brar.

¹⁰ Toennies, G., *Cancer Research*, 1947, 7, 193.

¹¹ Voegtlin, C., and Thompson, J. W., *J. Biol. Chem.*, 1926, 70, 801; Woodward, G. E., *Biochem. J.*, 1935, 29, 2405; Schenk, E. C., *Arch. f. exp. Path. u. Pharm.*, 1934, 175, 405.

¹² Brđicka, R., *Nature*, 1937, 139, 330; Walker, A. C., and Reimann, S. P., *Am. J. Cancer*, 1939, 37, 585; Winzler, R. J., and Burk, D., *J. Nat. Cancer Inst.*, 1944, 4, 417.

INSULIN ON GLUCOSE DEPENDENCE

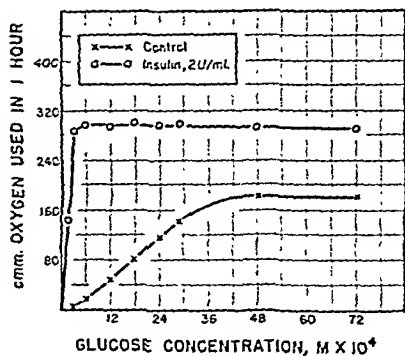


FIG. 2.

Zinc-insulin promoted alterations in the shape of the oxygen uptake-glucose concentration dependence curve.

Rice and Evans⁵ purport is a result of increased pyruvate dissimilation. *T. hippicum*, however, is incapable of oxidizing pyruvate, and the accumulation of pyruvate and the failure of malonate to moderate the response to insulin show that pyruvate oxidation was not involved. It is impossible to explain an increase in aerobic pyruvate production in the face of a decrease in glucose consumption, considering the stoichiometrical relationship between glucose and pyruvate in the parasite, without assuming that endogenous substrate is brought into the sequence. Again, this is the only means by which the enormous oxygen uptake and low concentrations may be reconciled. *T. hippicum* cannot respire upon endogenous substrate alone, nor have polysacchar-

ides been found in the organism,¹⁰ so that it would seem that insulin is making lipoid or proteinaceous materials available for dissimilation. It should be interjected, however, that these perturbations in the carbon balance were performed at glucose concentrations low enough that the endogenous contribution to the scheme would be proportionately significant and thereby detectable. Nevertheless, at glucose concentrations even as high as 0.044 M, where the endogenous contribution is proportionately small, 60-80% augmentation of oxygen uptake was yet evident. The slope of the insulin curve in Fig. 1 does not fall off as rapidly as that of the control, therefore, it seems that a stabilizing factor is at work as well as an augmenting one.

The discovery that insulin, contrary to the aerobic case, inhibited the anaerobic pyruvate production to an extent way out of proportion to the glucose sparing effect is no less startling than it is inexplicable. It may lead to the disturbing hypothesis that the aerobic and anaerobic pathways of pyruvate production are dissimilar.

Summary. Aerobically, insulin was found to greatly increase the oxygen assimilation and pyruvate production in *T. hippicum*, while at the same time the utilization of glucose was diminished.

Anaerobically, it was found to inhibit the production of pyruvate while the diminution in glucose consumption was of the same order as that under aerobic conditions.

TABLE I.
Specific Action of Zinc-Insulin on Oxygen-Uptake.

Added substances	Microliters oxygen used
Glucose, 0.028 M	187
Insulin, 2U/ml + glucose, 0.028 M	390
" 2U/ml	—3
Zn++, 0.005 mg/ml + glucose, 0.028 M	142
Cystine, 10 ⁻⁴ M + glucose, 0.028 M	180
*GS-SG, 10 ⁻⁴ M + glucose, 0.028 M	192
Tyrosine, 0.01 mg/ml + glucose, 0.028 M	173
Albumin, 1 mg/ml + glucose, 0.028 M	167
Insulin, 2U/ml + glucose, 0.028 M + malonate, 0.01 M	368

*Oxidized glutathione.

TABLE II.
Effect of Zinc-Insulin on Carbon Balance.*

	Insulin, 2U/ml		Control	
	Glucose used (micromoles)	Pyruvate formed	Glucose used (micromoles)	Pyruvate formed
Aerobic	1.86	7.50	2.85	5.67
Anaerobic	1.26	0.33	2.67	2.58

* Initial glucose concentration, 0.0011 M.

The specificity of the insulin action is shown in Table I. That the insulin itself was not being metabolized was indicated by the failure of the parasites to respire when glucose was withheld. Zinc in concentrations comparable to that provided by the zinc-insulin preparation did not increase the oxygen consumption and may have caused a slight inhibition. Disulfide groups were provided by cystine and oxidized glutathione in amounts approximating that found in the insulin. The data demonstrate that the action of the insulin cannot be attributed to its disulfide linkages *per se*, nor can it be attributed to a dynamic action of the tyrosine units in the molecule. That the effect was not a non-specific protein effect was tested by the addition of bovine serum albumin (fraction V, Armour) in a concentration roughly 10 times that of the insulin which it replaced. Malonate did not abolish the action of insulin, a finding which is compatible with the data of Table II in which it is shown that pyruvate oxidation was not promoted by the insulin.

Examination of Fig. 2 reveals that at a glucose concentration of 0.0012 M, for example, the oxygen utilized is about 4 times that which can be accounted for by the total amount of glucose available in the system

(3 ml volume), if one assumes that pyruvate is the final product.¹⁰ At lower concentrations the oxygen uptake cannot be accounted for by any possible metabolic scheme based upon glucose as the sole substrate. In order to determine what influence the insulin was exerting on the glucose-pyruvate relationship, these two substances were analysed from the experimental system. Table II presents data collected from aerobic and anaerobic experiments. Aerobically the glucose utilization was spared by insulin to the extent of 35%, but the pyruvate production was increased 32% over the controls. Anaerobically glucose utilization was diminished 53% in the presence of insulin, while there was a simultaneous diminution of 87% in pyruvate production.

Discussion. It is not obvious what general implications these actions of insulin may have regarding the mechanism of action of the hormone. In whole cell preparations of animal tissues the drug is generally considered to promote the disappearance of glucose. Insulin, however, was found to have no effect upon the hexokinase of the trypanosomes. Only in those instances cited has insulin been reported to affect the rate of oxygen uptake, which

¹⁰ Harvey, S. C., to be published.

TABLE I.
Inhibition of Sheep Cell Agglutinins in Infectious Mononucleosis Serum by Beef Stroma
Heated for 30 Minutes.

Temperature °C	Beef stroma dilutions			
	16,000	32,000	64,000	128,000
40	—	—	—	++++
60	—	—	—	++++
80	—	—	+	++++
100	—	—	++	++++

TABLE II.
Inhibition of Sheep Cell Agglutinins in Infectious Mononucleosis Serum by Beef Stroma
Digested by Trypsin or Pepsin.

Treatment of the beef stroma			Beef stroma dilutions
Pepsin at pH 3.0	2 days	37°C	1:128,000
" " "	3 "	" "	1:128,000
Control " " "	3 "	" "	1:128,000
Trypsin at pH 7.7	2 days	37°C	1:32,000
" " " "	5 "	" "	1:64,000
Control " " "	5 "	" "	1:64,000

TABLE III.
Inhibition of Sheep Cell Agglutinins in Infectious Mononucleosis Serum by Beef Stroma
Extracted with Organic Solvents.

Extraction	Extracted substance in %	Inhibition in dilutions	
		Extracted stroma	Extract
Acetone, room temperature	8.65	1:64,000	0
Alcohol, " "	20.0	1:16,000	1: 250
Alcohol, after acetone extraction, room temperature	12.6	1:16,000	1: 125
Ether, boiling	14.3	1:64,000	1:8000
Acetone, " "	15.8	1:64,000	0
Pyridine, 90°C, after acetone- and alcohol-extraction, at room temperature	4.9	1: 8,000	1:2000

and at the end of the experiment. From Table II it can be seen that neither trypsin nor pepsin digests the M.A. In view of these experiments, it appeared improbable that the M.A. of beef erythrocytes is a protein. Apart from the M.A., beef stroma also contains a rapidly digested protein-like antigen which will be reported in a later publication.

Attempts were made to extract the M.A. with cold and with boiling organic solvents. The extraction with cold liquids was carried out by stirring for several days, the extraction with boiling liquids by changing the solvent several times until the material was exhausted.

As can be seen from Table III, a small quantity of M.A. could be extracted by means of boiling ether or hot pyridine. How-

ever, since, contrary to the statements of Stuart, Griffin, Fulton, and Anderson,¹ the cold alcoholic extract also shows a small but definite activity, the possibilities of extraction by means of boiling ethyl alcohol were thoroughly investigated as described below. Beef stroma was extracted, first with acetone and then with cold 100% alcohol. On each occasion 50 cc solvent per g stroma were used and the suspension stirred for 2 days at room temperature.

In this way, we were able to remove about 20% almost inactive material. After this pretreatment with cold organic solvents the beef stroma was exhaustively extracted with boiling 100% alcohol under reflux. The solvent was changed several times and, in this way,

Isolation of the Heterogenetic "Mononucleosis Antigen" from the Stroma of Beef Erythrocytes.

H. SCHWARZWEISS AND J. TOMCSIK.

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Stuart and coworkers^{1,2} demonstrated the presence in beef erythrocytes of the heterogenetic "mononucleosis antigen" (M.A.) to which they gave the designation "B.T." ("beef thermostable antigen"). This antigen is contained in beef erythrocytes in a much greater concentration than in sheep erythrocytes. We have confirmed the findings of Stuart and coworkers, although we have not used the designation "B.T." since beef erythrocytes also contain other thermostable antigens which do not react with serum obtained from patients with infectious mononucleosis. These will be discussed in a later publication.

The previous method for determining serological activity consisted in measuring the decrease in the sheep cell hemagglutinin titer of a mononucleosis serum after adsorption by a thick suspension of erythrocytes. Our method, which we have already described in a previous communication,³ is as follows: 1. Determination of the sheep cell agglutinin titer of the serum using the centrifugation method. 2. Adsorption of the serum by various dilutions of antigen in geometric series, in such a way that the final dilution of the serum corresponds to two agglutinin-units. 3. Removal of the insoluble material by centrifugation and testing the clear supernatant liquid for sheep cell agglutination.

Using this method, we are in a position to carry out activity measurements on our preparations and to specify the dilution in terms of dry substance.

We use as starting material the stroma

¹ Stuart, C. A., Griffin, A. M., Fulton, Mc. D., and Anderson, E. G. E., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 209.

² Stuart, C. A., Griffin, A. M., Wheeler, K. M., and Battey, S., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 212.

³ Tomesik, J., and Schwarzweiss, H., *Schweiz. Z. Path. und Bakt.*, 1947, **10**, 407.

obtained from hemolysed erythrocytes by centrifugation with the Sharples supercentrifuge.

The yield from 100 cc of defibrinated blood is about 0.5 g stroma. The activity varied on different days between 1:8000 and 1:32,000 for sheep stroma and between 1:32,000 and 1:128,000 for beef stroma, the variation being principally dependent on the sheep erythrocytes used for agglutination. On the other hand, the degree of activity is influenced little by the nature of the patient's serum, provided that the serum is a typical infectious mononucleosis serum and that 0.1 cc of the adsorption mixture prepared according to our technic contains 2 agglutinating units.

In a previous investigation,⁴ we attempted to isolate the M.A. from sheep stroma. With pyridine and ethyl alcohol, etc., the Forssman antigen could be extracted while the M.A. remained behind in the stroma. Using N/30 NaOH or 1-2.5% NaHCO₃, it was possible to bring the M.A. with more or less success into solution, but without an increase in its serological activity.^{3,4}

In this work beef stroma was studied since this offers a very much better source of heterogenetic M.A.

As Table I shows, the serological activity of beef stroma towards the heterogenetic infectious mononucleosis antibody is but very little decreased even after heating to 100°C.

Digestion with trypsin and pepsin was carried out in either a phosphate or a citric acid-phosphate isotonic buffer solution. The pH values were controlled potentiometrically. 1 mg enzyme was used for each cc stroma suspension (1:250 dilution). The activity of the enzymes was controlled by the fact that a suspension of fibrin flakes was completely digested within 2 hours, both at the beginning

⁴ Schwarzweiss, H., and Tomesik, J., *Schweiz. Z. Path. und Bakt.*, in press.

efficiency of the method.

Table V shows that an activity of 1:1,000,000 can be reached starting from a mixed fraction possessing an initial activity of about 1:250,000.

After standing for one day at 4°C, the 80% alcohol extract with an activity of 1:1,000,000 gave a precipitate which was removed by centrifugation. The precipitate showed an activity of 1:80,000 and the material remaining in the solution an activity of 1:2,400,000. The last fraction, which was obtained as a brownish red powder after evaporating off the alcohol, was designated by us as a purified heterogenetic "mononucleosis antigen." It may be mentioned that we have attempted to extract the M.A. more readily from the stroma by saponification of the latter with sodium alcoholate (50 mg Na in 50 cc alcohol) under reflux. We found that this resulted in inactivation of the antigen.

We have attempted a further purification by treating the aqueous and the alcoholic solutions with activated charcoal both in the cold and at the boiling point. The charcoal effected complete decolorization, but, at the same time, the active substance also disappeared from the alcoholic solution. It could be detected in a suspension of the charcoal at a dilution of 1:8000 but could no longer be recovered.

Furthermore, we investigated the question whether M.A. is a necessary constituent of beef erythrocytes or whether as Sohler, Jaulmes, and Tissier⁵ presume, there are individual variations. Apart from numerous tests with mixed blood, from which we were always able to obtain the antigen, we have examined blood from three animals separately. In each case, we were able to obtain the antigen with correspondingly good activity. A further source of M.A. is sheep's blood stroma. From this, using the extraction technic described above, we were only able to

achieve activities of the alcoholic extract up to 1:8000.

Stuart, Griffin, Wheeler, and Battey² were able to demonstrate the presence of M.A. in rabbit serum by means of the hemolysis inhibition technic. Using our adsorption technic, we have examined dried rabbit organs: the heart, liver, spleen and cerebellum showed an activity of 1:250, the kidneys as much as 1:500, the stomach, small intestine, lungs and cerebrum only 1:64, and muscle was inactive. The heart, liver, spleen and kidneys were combined and extracted according to the scheme described. The cold acetone and alcohol extracts, as well as the boiling 100% alcohol fractions, were inactive. The boiling 80% alcohol extract exhibited an activity of 1:500 after 15 minutes and this rose to 1:4000 after extracting for 2 hours. As claimed by Stuart and his coworkers, the reason why the rabbit does not produce an antibody against M.A. fraction of beef erythrocytes is probably that it contains this antigen in its own organs. Thus, the heterogenetic M.A. occurs in beef and sheep erythrocytes as well as in rabbit organs. On the other hand, no trace of M.A. could be detected in rabbit erythrocytes, in peptone, in pepsin, or in trypsin preparations.

Summary. 1. From the stroma of beef erythrocytes it was possible to extract with boiling 80% alcohol the so-called heterogenetic "mononucleosis antigen," which, after purification, inhibits the sheep cell agglutination of the infectious mononucleosis serum in a dilution of 1:2,400,000. It is a thermostable hapten and is not digested by pepsin or trypsin.

2. The "mononucleosis antigen" which occurs in sheep stroma cannot be extracted with good activity by means of the technic effective for beef cells.

3. A serologically similar heterogenetic antigen could also be detected in rabbit organs, although in small quantities. This fact makes it comprehensible why the corresponding antibody cannot be artificially produced in rabbits.

⁵ Sohler, R., Jaulmes, Ch., and Tissier, M., *Ann. Inst. Pasteur*, 1945, 71, 463.

TABLE IV.
Inhibition of Sheep Cell Agglutinin in Infectious Mononucleosis Serum by Beef Stroma Extracted First with Acetone and Ethyl Alcohol at Room Temperature and Later with Boiling Ethyl Alcohol.

Extracted with	Fraction	Activity of the extract
Acetone, room temperature	0-4 days	0
Alcohol, " " " 100%	0-4 "	1:125
Alcohol, boiling, 100%	0-15 min.	1:32,000
" " "	15-30 "	1:16,000
" " "	30-60 "	1:8,000
" " "	60-120 "	1:4,000
Alcohol, boiling, 80%	0-15 min.	1:1,000,000
" " "	15-30 "	1:250,000
" " "	30-60 "	1:500,000
" " "	60-120 "	1:250,000
" " "	2-4 hr	1:500,000
" " "	4-8 "	1:32,000
Stroma residue		1:8,000

TABLE V.
Purification of a Less Active Beef Stroma Extract. (Initial dry weight 1.7 g.).

Extraction	Extracted substance in %	Inhibition of Sheep cell agglutination
Boiling acetone, 179 cc 0-15 min.	21.0	0
" " " " 15-30 "		
" " " " 30-60 "		
Boiling 100% alcohol 85 cc 0-15 min.	16.2	1:4,000
" " " " 15-30 "	6.75	1:8,000
" " " " 30-60 "	2.0	1:8,000
" " " " 60-120 "	1.6	1:8,000
Boiling 80% alcohol 85 cc 0-15 min.	28.0	1:1,000,000
" " " " 15-30 "	4.55	1:320,000
" " " " 30-60 "	0.7	1:480,000
" " " " 60-120 "	0.95	1:320,000
Residue	29.2	

various fractions were obtained. After completion of the exhaustive extraction with 100% alcohol, which removed about 0.3-0.7% dry substance calculated with reference to the stroma, extraction under reflux was performed with 80% alcohol. The fraction so obtained contained the M.A. in a dissolved form and in very much higher activity than in the stroma itself.

Table IV shows that, using 100% alcohol, the M.A. isolated has only poor activity, but that, when 80% alcohol is used, the activity jumps to 1,000,000. Likewise, a drop in the activity of the stroma residue is manifest. Altogether 5.1% dry substance can be extracted from the stroma, with 80% boiling alcohol. This value shows a certain variation

dependent upon the stroma preparation and upon the conditions of the experiment.

From numerous experiments we obtained fractions of high activity (1:256,000-1:1,000,000) and low activity (below 1:256,000), which we sometimes combined.

Further purification of the two fractions was achieved by the following procedure:

The alcohol was distilled off under reduced pressure the residue evaporated to dryness on the water bath and then exhaustively extracted under reflux, first with acetone, changing the solvent several times, then with 100% alcohol and finally with 80% alcohol.

In this way, a small quantity of highly active substance could be recovered from the fraction with low activity, an indication of the

TABLE I.
Inhibition of Sheep Agglutinins in Serum Sickness, Infectious Mononucleosis, and Forssman Serum by Various Agents.

Antigens	Inhibition titer of the antigens toward hemagglutinins in		
	Serum sickness	Inf. mononuel.	Forssman serum
Sheep erythrocyte stroma	6,000	24,000	32,000
Beef " "	64,000	64,000	0
Horse " "	4,000	8,000	0
" " "	120*	320*	0
" serum	4*	0	0
Rabbit erythrocyte stroma	250	0	0
" " "	500*	0	0
" " hemolyzed	trace	0	0
" spleen and kidney	16,000	250	0
" liver	2,000	250	0
" muscle	2,000	0	0
Guinea pig kidney	4,000	0	1,200
Alcoholic extr. of g. pig kidney	4,000	0	16,000
Several peptones	0	0	0

0 = no inhibition in a dilution 1 : 62.5, calculated in terms of dry weight of stroma or of various organs, or in 10% suspension of erythrocytes.

* = the dilutions were calculated exceptionally in volumes and not in weight of the dry substance.

1:48, *i.e.*, 0.1 cc of the serum diluted 1:16 still completely agglutinated 0.2 cc of a 0.75% suspension of sheep red blood corpuscles. The type of hemagglutinin was determined by examining all the sera with various dilutions of the following antigens: 1. Sheep blood stroma. 2. Cattle blood stroma. 3. Guinea pig kidney. 4. Rabbit erythrocytes. The hemagglutinins were considered to be characteristic for horse serum sickness when they were adsorbed by all 4 antigens. Of 9 adult sera, which showed a high agglutinin titer, 7 sera were found to be typical for horse serum sickness on the basis of their adsorption, while of 8 sera obtained from children only 2 were found to be typical for horse serum sickness. The serum HD 85, which was selected as serological indicator for the subsequent work, agglutinated sheep red blood corpuscles at a dilution of 1:240, cattle blood corpuscles at 1:120, and hemolysed both types of blood corpuscles at a dilution of 1:120. The serological results summarized in Table I are the average values of several determinations.

In assessing the results shown in Table I it is to be noted that the difference between a negative and a positive agglutination was very easy to determine in the case of the infectious mononucleosis and the Forssman sera, whereas the transition from a negative to a positive

reaction after adsorption of the serum sickness serum took place stepwise, with the result that the titer of the antigen with the latter serum exhibited fluctuations of more than 50% in different readings.

As can be seen from Table I, the beef stroma exhibited the highest serological activity of all antigens examined, both with mononucleosis and with serum sickness serum. Since boiling the aqueous stroma suspension is not detrimental to the serological activity, it may be concluded that the B.T. (beef thermostable substance) described by Stuart, Griffin, Wheeler, and Battey⁵ contains both M.A. and S.S.A. On the other hand, as already known, guinea pig kidney contains the Forssman antigen and S.S.A. Thus, if our conception described above is correct, it should be possible to isolate S.S.A. both from beef stroma and from guinea pig kidney. Since we were able, as previously reported,¹⁰ to isolate the heterogenic mononucleosis antigen in a purified form from B.T. by fractional extraction with cold and boiling organic solvents, we also employed the same procedure for the isolation of S.S.A.

Table II shows that we were able by means of 100% boiling alcohol to extract S.S.A. from beef stroma with a considerable increase in its activity. Since, as shown in a previous study,¹⁰ a considerable increase in the M.A.

Nature of the Heterogenetic Hapten Reacting with Hemagglutinins in Horse Serum Sickness.

J. TOMCSIK AND H. SCHWARZWEISS.

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An increase in the sheep's blood hemagglutinins in horse serum sickness has been described by both Hanganutziu and Deicher. Paul and Bunnell have described a similar phenomenon in infectious mononucleosis. It has subsequently been reported in numerous publications that the hemagglutinins in serum sickness and in infectious mononucleosis correspond to two different heterophilic antibodies (e.g., Davidsohn *et al.*,¹⁻³ Stuart *et al.*⁴⁻⁶ Schiff⁷ even referred to a serum sickness antibody and gave the name serum sickness antigen (S.S.A.) to the receptor which reacts with the hemagglutinin in serum sickness. For the sake of simplicity, this expression will be retained in the present publication, although it should be noted that the part played by this antigen in the genesis of serum sickness has been in no way confirmed.

According to our conception, it is justifiable to include in the group of heterogenetic antigens, in addition to the Forssman antigen, both S.S.A. and the erythrocyte-antigen reacting with the antibodies in infectious mononucleosis (M.A.) as separate antigens. However, this conception does not appear to be a general one. For example, Boyd⁸ writes: "The Forssman antigen is probably not a definite chemical entity, but a serological conception, a collective term covering substances

which, injected into rabbits produce sheep hemolysins. The original Forssman antigen was a concept somewhat more narrow." In contrast to this conception, we consider it more correct to retain the original precise definition of the Forssman antigen and to recognize the other heterogenetic antigens mentioned as different substances. However, this conception presupposes a mosaic-like structure of certain cell antigens, the individual antigens being separable from one another even when they are components of a large molecule. The purpose of this work was to separate the S.S.A. from the other heterogenetic antigens described above, even when they occur in the same cell, and to elucidate its nature.

The technics for carrying out the hemagglutination and the inhibition reactions were described in previous papers.^{9,10} When the nature of the hemagglutination is not more precisely defined, sheep's blood agglutination is to be understood. By degree of activity, we understand the reciprocal value of the highest dilution of antigen, calculated in terms of dry substance, which is capable of completely suppressing the hemagglutinating power of two antigen units.

For the purpose of selecting a human serum to serve in the subsequent course of the work as a serological indicator for the study of the S.S.A., 26 human sera were examined. The shortest interval of time between injection of the horse serum and removal of a sample of blood was 7 days. In 16 cases, the serum was obtained from more or less severe cases of serum sickness with exanthema. In 17 cases, the agglutination titer was more than

¹ Davidsohn, J., *J. Immunol.*, 1929, **16**, 259.

² Davidsohn, J., *J. Immunol.*, 1930, **18**, 31.

³ Davidsohn, J., *J. Infect. Dis.*, 1933, **53**, 219.

⁴ Stuart, C. A., Tallman, J., and Brintzenhoff, E., *J. Immunol.*, 1935, **28**, 85.

⁵ Stuart, C. A., Griffin, A. M., Wheeler, K. M., and Battey, S., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 212.

⁶ Stuart, C. A., Fulton, Mc. D., Ash, R. P., and Gregory, K. K., *J. Infect. Dis.*, 1936, **59**, 65.

⁷ Schiff, F., *J. Immunol.*, 1937, **33**, 305.

⁸ Boyd, W. C., *Fundamentals of Immunology*, Interscience Publishers, 1947, 2nd ed., p. 141.

⁹ Tomcsik, J., and Schwarzweiss, H., *Schweiz. Z. Path. und Bakt.*, 1947, **10**, 407.

¹⁰ Schwarzweiss, H., and Tomcsik, J., *Proc. Soc. Exp. Biol. and Med.*, accompanying paper.

occurs heterogenetically in guinea pig kidney and it could also be isolated from the Forssman antigen although without increase in activity.

3. Horse serum is a poorer source for isolation

of the so-called serum sickness antigen. The latter could be isolated from the serum, after removal of the albumin bodies by coagulation with heat, with an activity of 1:1,500.

16788 P

Dimethyl Ether of *d*-Tubocurarine Iodide as an Adjunct to Anesthesia.

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The capacity of curare to block efferent nervous impulses at the myoneural junction has long been known. Bennett¹ administered curare clinically to minimize the trauma to patients undergoing convulsive shock therapy. Subsequently, Griffith and Johnson,² and Cullen³ reported the use of curare as an adjunct for relaxing the abdominal muscles during inhalation anesthesia.

The dosage of *d*-tubocurarine chloride administered to patients during anesthesia rarely produces toxic cerebral or cardiovascular manifestations. The drug frequently produces moderate or severe respiratory depression. For this reason, other natural and synthetic compounds have been investigated. One of the more promising preparations is dimethyl ether of *d*-tubocurarine iodide.^{4,5} This compound has a chemical formula $C_{40}H_{18}O_6N_2I_2 \cdot 3H_2O$. It will be referred to as M-curare. In animal experiments, Chen and Swanson⁶ demonstrated that M-curare effectively decreases muscle tone without producing respiratory depression.

Experimental. We have administered M-

¹ Bennett, A. E., *Am. J. Med. Science*, 1941, 202, 101.

² Griffith, H. R., and Johnson, G. E., *Anesthesiology*, 1942, 3, 418.

³ Cullen, S. C., *Surgery*, 1943, 14, 261.

⁴ King, H., *J. Chem. Soc., London*, 1935, (pt. 2), 1381-1389.

⁵ Collier, H. O., and Paris, S. K., *Nature*, 1948, 161, 817.

⁶ Chen, K. K., and Swanson, E. E., personal communication of unpublished data.

TABLE I.

Anesthesia	No. of patients	Range of initial dosage in mg		
		Min.	Max.	Avg
Cyclopropane	16	1	4	2
Ether	49	1	5	2.5
Nitrous oxide	35	1	6.5	3

curare to anesthetized patients of both sexes, ranging in age from 10 to 84 years of age. The degree of relaxation reported was based on the evaluation of the surgeon.

The drug was dissolved in a distilled water and the curare adjusted to .5 mg per cc.* A preservative was added. The pH varied from 4.0 to 5.0. The solutions were stored at room temperature and used over a period of several weeks. Different lots of the drug were administered by the intravenous route. The initial dose consisted of 1 mg or more of the drug.

Data presented in the table show the amount of M-curare required to produce adequate relaxation. The dosage recorded was given in one or more injections within a period of 10 minutes.* Adequate relaxation was not noted in any patients who received less than 1 mg of M-curare.

It will be noted that an average of 2 mg of M-curare produced satisfactory relaxation in 16 patients receiving cyclopropane anesthesia. An average of 2.25 mg was required to produce adequate relaxation with ether

* Supplied by Eli Lilly Research Laboratories, Indianapolis, Ind.

TABLE II.

Inhibition of Sheep Agglutinins in Infectious Mononucleosis and Serum Sickness Serum by Beef Stroma Extracted First with Acetone and Ethyl Alcohol at Room Temperature and Later with Boiling Ethyl Alcohol.

Extracted with	Fraction	Inhibition titer of the extracts toward hemagglutinins in	
		Inf. mononuel.	Serum sickness
Acetone, room temperature	0- 4 days	0	0
Alcohol, " " " 100%	0- 4 "	125	16,000
Alcohol, boiling, 99.9%	0- 4 hours	16,000	192,000
" " " "	4- 8 "	48,000	256,000
" " " "	8-12 "	48,000	256,000
Alcohol, boiling, 80%	0- 4 hours	1,100,000	6,000
" " " "	4- 8 "	500,000	48,000
" " " "	8-12 "	256,000	32,000

activity can be detected only in the fractions obtained with 80% boiling ethyl alcohol, it was possible to separate the two heterogenetic antigens from B.T. by means of a simple procedure based on their difference in solubility. After repeated application of this procedure, two fractions were obtained from beef stroma which exhibited the following activities:

Fraction A: S.S.A. activity 512,000, M.A. activity 8,000.

Fraction B: S.S.A. activity 16,000, M.A. activity 1,000,000.

Preparation of pure S.S.A. from rabbit stroma was not attempted as its activity was too small. It is all the more difficult to give an explanation of the small serological activity of this stroma in comparison with the intact or boiled rabbit erythrocyte suspension since the hemolysed erythrocytes were completely inactive; thus, the active substance of the stroma could not be detected outside the stroma.

The next question was whether the S.S.A. could be separated from the Forssman antigen of guinea pig kidney. 10 g dried and finely powdered guinea pig kidney were extracted twice, for 9 hr each time, with 250 cc 97% alcohol in a Soxhlet apparatus. Afterwards the residue was further extracted for varying lengths of time, up to 3 hours, with boiling 97% alcohol. Both antigens were detectable in the cold alcoholic extract, but when the already thoroughly extracted residue was further extracted with boiling

alcohol, only S.S.A. with an activity of 1:1,000 could be detected. In contrast to beef stroma, it was thus possible to isolate the S.S.A. from guinea pig kidney, but without elevation of the serological activity.

After these investigations of its heterogenetic occurrence, S.S.A. was finally investigated in the original antigen, *i.e.*, in horse serum. 750 cc horse serum with a S.S.A. activity of 1:2 were coagulated at 100° and the coagulated mass of albumin separated from the liquid portion on a Buchner filter. Both fractions were dried in a vacuum drying oven. The suspension of coagulated albumin was still inactive against serum HD 85 in a dilution of 1:50, while the dried residual substance obtained from the filtrate showed an activity of 1:1,200. By fractional extraction with alcohol, however, no increase in activity could be achieved. Both the cold and the hot alcoholic extracts had a serological activity of about 1:1,500.

Summary. 1. From beef stroma pretreated at room temperature with acetone and alcohol, a fraction was isolated with boiling 100% alcohol which, in a dilution of 1:500,000, combines with the sheep blood agglutinin of human serum produced during serum sickness. Using the terminology of Schiff, this fraction corresponds to the heterogenetic serum sickness antigen; it could be separated to a large extent from the heterogenetic mononucleosis antigen which also occurs in beef stroma.

2. The so-called serum sickness antigen also

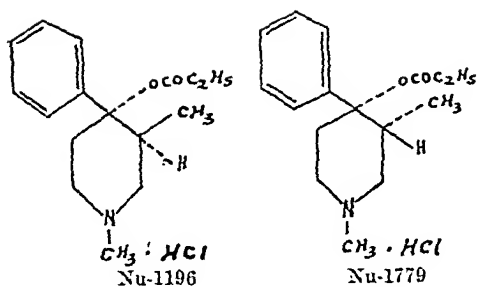


Fig. 1.

demerol, and methadon exert their effects on oxidative processes in a manner similar to the anesthetics and hypnotics.

In the following investigation two new analgetic agents,⁵ namely, (a) *dl*-α-1,3-dimethyl-4-phenyl-4-propionyloxy-piperidine hydrochloride (Nu-1196), and (b) *dl*-β-1,3-dimethyl-4-phenyl-4-propionyloxy-piperidine hydrochloride (Nu-1779)* were compared with methadon in regard to their *in vitro* effects on brain metabolism using the conventional manometric methods.^{6,7} The formulae for these compounds are given in Fig. 1.

Randall and Lehman⁸ have made studies on these compounds relative to analgesia in the rat. They report that Nu-1196 has about the same analgetic potency as morphine and Nu-1779 is about 5 times as active as morphine in rats. Studies⁹ carried out in our laboratory indicate both of these new agents to be potent analgetics in normal subjects.

¹ Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, 1932, **26**, 725.

² Jowett, M., *J. Physiol.*, 1938, **92**, 322.

³ Fuhrman, F. A., and Field, J., 2nd., *J. Pharm. and Exp. Therap.*, 1943, **77**, 392.

⁴ Elliott, H. W., Warrens, A. E., and James, H. P., *J. Pharm. and Exp. Therap.*, 1947, **91**, 98.

⁵ Ziering, A., and Lee, J., *J. Org. Chem.*, 1947, **12**, 911.

* These drugs were supplied by the Hoffmann-LaRoche, Inc., Nutley, N. J.

⁶ Dixon, M. M., *Manometric Methods*, Macmillan Co., New York, 1943.

⁷ Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques*, Burgess Publ. Co., Minneapolis, Minn., 1945.

⁸ Randell, L. O., and Lehmann, G., *J. Pharm. and Exp. Therap.*, 1948, **93**, 314.

⁹ Gross, E. G., Holland, H. L., and Schueler, F. W., *J. Applied Physiol.*, in press.

Nu-1196, while being the least potent on a milligram basis, shows the greatest clinical promise since at comparable analgetic levels its side effects are minimal relative to Nu-1779 and morphine.

Procedure and Results. The oxygen consumption of brain cortex tissue slices (Rat) respiring in the presence of various substrates together with the three drugs Nu-1196, Nu-1779, and methadon were determined by the direct method of Warburg.

Cerebral cortex slices were cut with a razor and template¹⁰ and the tissues were suspended in a modified Krebs-Ringer solution. The modified medium used was prepared according to the directions given by Elliott *et al.*⁴ Phosphate buffer was used in all of the oxygen uptake measurements together with a gas phase of oxygen. After 2½ to 3 hours (*i.e.*, after at least 90% decrease in the initial O₂ uptake) the appropriate substrate was added. The shaking was continued for one hour after addition of the substrate. The drugs were made up in Ringer's solution and added to the tissues from the side arm after the control period of 60 minutes with substrate and continued for an additional 60-minute period. Each vessel, therefore, served as its own control and in addition controls were run for the entire 120-minute period.

Table I summarizes the experimental results expressed as per cent gross inhibition of oxygen consumption during the second hour over that obtained during the first hour. We have also summarized the oxygen uptake values in this table in terms of the mm³ O₂ uptake per milligram of wet weight tissue per hour, due to the added substrate with and without drug. Table II summarizes the effect of the analgetic agents upon the oxygen uptake of brain after at least 90% decrease in the oxygen consumption where no substrate has been added. The per cent net inhibition of oxygen consumption due to drug alone was calculated by subtracting the per cent fall of the control during its second hour over its first hour from the respective per cent gross inhibitions due to the drugs.

¹⁰ Crimson, J. M., and Field, J., 2nd., *Am. J. Physiol.*, 1940, **130**, 231.

anesthesia. With nitrous oxide anesthesia, the comparable dosage was 3 mg.

Relaxation produced by the initial dose of M-curare sufficed for surgical procedures, lasting 60 to 90 minutes. After this period, supplemental injections of .5 to 1 mg were infrequently required. The anesthesia level was maintained in lower plane I or upper plane II in all the patients. Reflexes were absent in 8 patients at the termination of surgery. Intubation was performed in 44 patients where the type of surgery made the technic mandatory.

No cardiovascular changes were noted in any of the patients. In no case could a fall in blood pressure be attributed to the drug.

Mild respiratory depression was observed in 9 patients in a series of 100 cases. In 7 of these, respiratory depression existed before surgery as the result of excessive premedication. One of the remaining cases received 7.5 mg of M-curare during a 15-minute period. Five minutes after the final injection, the rate of respiration decreased from a normal of 22 per minute to 14. Ten minutes later it had returned to the normal rate. Subsequently, the patient received injections of .5 mg and 1 mg without the development of respiratory embarrassment. Mild respiratory depression occurred in the other patient 3 minutes after the injection of 1.5 mg of M-curare. The rate of respiration decreased from a normal of 24 per minute to 16. This depression persisted for 5 minutes and the rate of respiration returned to a normal of 24. Subsequent injections of .5 mg and 1 mg of

M-curare caused no untoward respiratory changes. Each of these patients maintained an adequate tidal exchange during the period of respiratory depression.

Discussion. The relaxation obtained with M-curare in this study was comparable to that obtained by other workers using *d*-tubocurarine chloride. The drug appears to have a selective action on skeletal muscle similar to that of *d*-tubocurarine chloride, but seldom affects the muscles of respiration.

No gross or microscopic changes were observed by Chen and Swanson⁶ in any of the organs of the experimental animals which received lethal doses of M-curare. The median lethal dose (LD_{50}) of M-curare in rabbits by intravenous injection, plus and minus standard error, was found to be 0.031 ± 0.002 mg per kg of body weight. The average dose sufficient to produce head drop in rabbits was approximately 0.0167 mg per kg of body weight (usually referred to as a rabbit unit). A group of 8 rabbits tolerated one rabbit unit per day for 10 days. At the end of this period, 6 out of 8 rabbits succumbed to an LD_{50} injected intravenously. This gave evidence that neither tolerance nor cumulation had developed.

Summary. The administration of the dimethyl ether of *d*-tubocurarine iodide (M-curare) to 100 patients produced relaxation comparable to that of *d*-tubocurarine chloride. Respiratory depression was seldom observed and was of minor degree when present. Less M-curare than *d*-tubocurarine chloride is needed on a mg-for-mg basis.

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Effect of Two New Analgetic Agents on the Oxygen Consumption of Brain *In vitro*.

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Quastel and others^{1,2,3} have conducted investigations on the actions of hypnotics and anesthetic agents on brain metabolism in which they have indicated parallels to exist

between *in vivo* and *in vitro* activity. Similar studies by Elliott, Warrens, and James⁴ suggest that, except for their findings on succinate oxidation, there is no evidence that morphine,

TABLE II.
Effect of Analgetic Agents on Oxygen Uptake of Brain After at Least 90% Decrease* in Respiration on Endogenous Substrates.

	Average mm ³ O ₂ uptake per hr per mg wet weight of tissue			
	No drug.	1st hr No added substrate	With drug.	2nd hr No added substrate
Morphine 0.01M		.167		.019
Methadon 0.01M		.133		.029
Nu-1779 0.01M		.102		.042
Nu-1196 0.01M		.074		.011
Control		.161		.073

* Drug was added after 2½ - 3 hr of shaking in Warburg flasks. No substrate was added.

The effects produced by the drugs are compared at equi-molar concentrations with methadon (at 0.01M) on glucose as a reference substrate. This concentration of methadon was found experimentally to produce a significant inhibition of the oxygen consumption of brain respiring on glucose.⁴

Conclusion. Our experiments indicate that significant inhibitions of the oxygen consumption of brain slices may be obtained using the new analgetic agents Nu-1196 and Nu-1779 when this tissue respire on glucose, lactate, pyruvate, α - and β -glycerophosphate (52% α), citrate, and possibly succinate. It must be pointed out, however, that inhibitions

due to drug with citrate as a substrate are to be considered quite dubious since the normal increase in O₂ consumption by this substrate alone is so small. Doubtful inhibitions were produced when brain slices respired on glutamate and oxalacetate. Nu-1196 and Nu-1779 showed insignificant effects at concentrations less than 0.01M.

As in the case of demerol, methadon, and morphine reported by Elliott *et al.*,⁴ the concentrations of the new agents Nu-1196 and Nu-1779 required to produce significant inhibitions were vastly larger than that required in the production of *in vivo* effects.

16790

Reduction in the Number of Adult *Trichinella spiralis* in Rats After Treatment with Naphthoquinones.*

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The effect of naphthoquinones on the metabolism of *Schistosoma mansoni* has been studied recently.¹ 2-methyl-1,4-naphthoquinone inhibits the rate of glycolysis of *S. mansoni* *in vitro*. Administration of this compound with subcurative doses of "Fuadin" to

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

¹ Bueding, E., Peters, L., and Waite, J. F., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 111.

mice infected with *S. mansoni* causes a marked decrease in the rate of glycolysis of the worms. Other naphthoquinones varied as to the degree of their anti-glycolytic activity *in vitro* as well as *in vivo*. The inhibitory effect on glycolysis manifested by these naphthoquinones is decreased markedly when the worms are incubated in dialysed blood serum instead of a protein-free salt solution. This suggests that interaction of the naphthoquinones with serum proteins would reduce any intrinsic activity of these compounds

TABLE I.
 Inhibition of the Oxygen Uptake of Brain Slices by Nu-1196 and Nu-1779.

Substrate and drug	No. of flasks N	Δ (net change) in O_2 uptake due to the presence of the given substrate in the absence and in the presence of the drug		% Gross inhibition $m \pm \sigma_m$	% Net inhibition $M \pm \sigma_M$
		First hr* without drug	Second hr† with drug		
Glucose 0.2%					
Methadon .01M	4	1.34	0.24	82.5 ± 1.2	74.7 ± 1.5
Nu-1779 .01M	8	1.43	0.88	48.0 ± 4.2	41.1 ± 4.4
Nu-1196 .01M	4	1.46	0.76	50.0 ± 5.5	42.2 ± 5.6
Control (no drug)	9	1.57	1.53	7.8 ± 1.1	
Glucose 0.3%					
Morphine HCl .01M	8	1.76	1.60	10.1 ± 5.2	2.1 ± 5.3
Control (no drug)	6	1.96	1.79	8.0 ± 1.2	
Lactate 0.2%					
Methadon .01M	11	0.40	0.31	68.7 ± 3.9	44.1 ± 5.9
Nu-1779 .01M	9	0.45	0.37	57.6 ± 6.3	33.0 ± 7.7
Nu-1196 .01M	12	0.44	0.24	51.0 ± 3.9	26.4 ± 5.9
Control (no drug)	9	0.44	0.41	24.6 ± 4.5	
Succinate 0.2%					
Methadon .01M	10	0.63	0.36	43.8 ± 3.2	19.5 ± 5.4
Nu-1779 .01M	10	0.62	0.49	36.9 ± 2.7	12.6 ± 5.1
Nu-1196 .01M	11	0.62	0.40	35.3 ± 2.5	11.0 ± 5.0
Control (no drug)	7	0.59	0.37	24.3 ± 4.3	
Pyruvate 0.2%					
Methadon .01M	7	0.47	0.11	74.9 ± 2.8	58.1 ± 5.3
Nu-1779 .01M	7	0.38	0.10	72.5 ± 2.9	55.7 ± 5.4
Nu-1196 .01M	7	0.36	0.11	69.3 ± 1.6	52.5 ± 4.8
Control (no drug)	6	0.36	0.30	16.8 ± 4.5	
Citrate 0.2%					
Methadon .01M	7	0.26	0.05	73.0 ± 1.3	39.0 ± 4.4
Nu-1779 .01M	6	0.20	0.03	86.2 ± 5.0	52.2 ± 6.5
Nu-1196 .01M	8	0.22	0.08	81.0 ± 2.1	47.0 ± 4.7
Control (no drug)	6	0.25	0.04	34.0 ± 4.2	
$\alpha + \beta$ -glycerophosphate .2% (52% α)					
Methadon .01M	4	0.40	0.32	49.0 ± 3.2	34.0 ± 7.3
Nu-1779 .01M	4	0.30	0.19	30.0 ± 8.8	15.0 ± 11.0
Nu-1196 .01M	4	0.34	0.17	18.2 ± 4.1	32.0 ± 7.7
Control (no drug)	4	0.34	0.26	15.0 ± 6.6	
Glutamate .2%					
Methadon .01M	4	0.28	0.07	58.0 ± 2.8	0.0 ± 4.2
Nu-1779 .01M	4	0.25	0.07	68.0 ± 1.9	10.0 ± 3.7
Nu-1196 .01M	4	0.25	0.10	74.0 ± 3.4	16.0 ± 4.7
Control (no drug)	4	0.28	0.11	58.0 ± 3.2	
Oxalacetate .2%					
Methadon .01M	4	0.35	0.14	62.5 ± 4.3	-0.5 ± 5.7
Nu-1779 .01M	4	0.32	0.23	44.0 ± 1.9	-10.0 ± 4.1
Nu-1196 .01M	4	0.36	0.13	62.0 ± 1.8	-1.0 ± 4.2
Control (no drug)	4	0.33	0.12	63.0 ± 3.7	

m = Mean % gross inhibition.

σ_m = Standard error of mean % gross inhibition.

M = Mean % net inhibition.

σ_M = Standard error of mean % net inhibition.

M = m experiment — m control.

$\sigma_M = \sqrt{\sigma_m^2 \text{ experiment} + \sigma_m^2 \text{ control}}$

* Net average mm³ O_2 uptake per hour per mg wet weight of tissue due to added substrate.

† Net average mm³ O_2 uptake per hour per mg wet weight due to added substrate after addition of drug.

cleus. Several of the compounds that had no effect on adult *T. spiralis* are more effective inhibitors of glycolysis of *S. mansoni* than 2-methyl-1,4-naphthoquinone.² On the other hand, the anti-glycolytic activity of 2-hydroxy-3-piperidinomethyl-1,4-naphthoquinone against glycolysis of schistosomes was a hundred times lower than that of 2-methyl-1,4-naphthoquinone.¹ Apparently, two different mechanisms are involved in the effect of naphthoquinones against adult trichinae and on glycolysis of schistosomes, since compounds that possess high anti-glycolytic activity did not reduce the number of trichinellae and the reverse was true for the 2-hydroxy-3-piperidinomethyl derivative.

No indication was obtained as to whether 2-methyl-1,4-naphthoquinone inhibits any specific metabolic reaction of adult trichinel-

lae. The metabolism at the adult stage of this parasite differs markedly from that of the larvae. Unlike the latter,³ adult trichinellae have an extremely low rate of oxygen uptake and do not contain any significant amount of glycogen. Furthermore, it was found that the adult worms do not utilize glucose present in the medium and do not produce any significant amounts of lactic acid or other acids when incubated in a salt medium containing glucose.

Summary. The oral administration of 2-methyl-1,4-naphthoquinone and 2-hydroxy-3-piperidinomethyl-1,4-naphthoquinone to rats infected with *Trichinella spiralis* produced a significant reduction in the number of intestinal adult trichinellae. Nine other structurally related naphthoquinones had no such effect.

² Bueding, E., and Peters, L., unpublished observations.

³ Stannard, J. W., McCoy, O. R., and Litchford, W. B., *Am. J. Hyg.*, 1938, 27, 666.

16791

Activity of Pantothenol as Pantothenic Acid in Promoting Chick Growth.*

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The activity of pantothenol as pantothenic acid in various species has been recently reviewed.¹ It is of interest that this compound interferes with the utilization of pantothenic acid by certain microorganisms² whereas in rats³ and human beings^{4,5} it is converted into pantothenic acid and appears to be as active

as pantothenic acid itself in these two species. From the results presented in this paper, it is concluded that on an equivalent basis, molecule for molecule, pantothenol is 86% as active as pantothenic acid in promoting chick growth.

Nine groups of white leghorn cockerels one week of age were fed the low pantothenic acid diet previously described.⁶ After 4 days of depletion, graded doses of calcium pantothenate ranging from 16 to 160 μ g per chick per day were administered to 6 of the groups by pipette placed directly in the crop. The remaining 3 groups received pantothenol in the same manner at 40, 65, and 100 μ g per

* Supported in part by grants-in-aid from the Nutrition Foundation, Inc., New York City, the Milbank Memorial Fund, New York City, and Swift & Company, Chicago, Ill.

¹ Anonymous, *Nutrition Rev.*, 1948, 6, 272.

² Snell, E. E., and Shive, W., *J. Biol. Chem.*, 1945, 158, 551.

³ Pfalz, H., *Z. f. Vitaminforsch.*, 1943, 13, 236.

⁴ Burlet, E., *Z. f. Vitaminforsch.*, 1944, 14, 318.

⁵ Rubin, S. H., Cooperman, J. M., Moore, M. E., and Scheiner, J., *J. Nutrition*, 1948, 35, 499.

⁶ Hegsted, D. M., and Lipmann, F., *J. Biol. Chem.*, 1948, 174, 89.

TABLE I.
Number of Adult Trichinae Recovered from Rats After Treatment with a Single Oral Dose of 200 mg of Various 1,4-quinones.

Compound	No. of rats used		Avg No. of worms recovered	
	Treated	Untreated	Treated	Untreated
2-Methyl-1,4-naphthoquinone	5	2	49	250
" "	5	2	217	689
" "	5	2	76	461
" "	10	3	39	411
" "	10	3	88	615
2-Hydroxy-3-N-piperidinomethyl-1,4-naphthoquinone	2	2	100	518
2-Hydroxy-3-N-piperidinomethyl-1,4-naphthoquinone	5	2	68	507
2-Hydroxy-3-N(2-methylpiperidino)-methyl-1,4-naphthoquinone	2	2	483	467
3-Hydroxy-2-isoamyl-naphthoquinone	2	2	412	510
3-Hydroxy-2-methyl-octyl-1,4-naphthoquinone	2	2	361	366
2,3-Dichloro-1,4-naphthoquinone	2		508	
Tolu-p-quinone	2	2*	581	604*
Beta-carbomethoxyethyl-(2-methyl-3-n-thiobutyl-1,4-naphthoquinonyl-6)-ketone	2		388	
2-Methyl-3-thioethyl-6-butyryl-1,4-naphthoquinone	2		444	
3-N-Thioamyl-2-chloro-1,4-naphthoquinone	2	2*	319	420*
2,5-ditertiary-butyl-1,4-hydroquinone	2		480	

* The same two rats served as controls for the accompanying three drugs tested.

against parasites living in a habitat with a high protein content. Of interest, therefore, is the study not only of their therapeutic utility, but also of the effect of naphthoquinones on the metabolism of intestinal worms, since these live in a medium relatively low in protein. The following is a report of the results obtained after treating rats with naphthoquinones during the intestinal stage of infection with *Trichinella spiralis*.

Rats weighing from 200 to 300 g were fed by stomach tube with 1100 to 1500 infective trichinae larvae each. The larvae were obtained from trichinous rat meat digested in a pepsin-hydrochloric acid mixture. Twenty-four hours later the rats were given a single dose of 200 mg[†] of the compound to be tested.[‡] The drug was suspended in mucilage

of tragacanth and then fed through a stomach tube. On the fifth day after infection the rats were killed and the number of adult trichinae present in small and large intestine were recovered and counted. Untreated rats were killed together with the treated ones to serve as controls.

Of the 11 compounds tested only two (2-methyl-1,4-naphthoquinone and 2-hydroxy-3-piperidinomethyl-1,4-naphthoquinone) caused a diminution in the number of intestinal trichinae (Table I). The average number of worms recovered in the animals treated with these two compounds was significantly less than in the untreated ones. The chi-square test was calculated for each drug and the values of *P* were beyond the 1% level of significance.

It is of interest that the reduction in the number of worms was caused by only two of the naphthoquinones tested. Since all the compounds are closely related, the effect is apparently determined by highly specific groups attached to the naphthoquinone nu-

[†] For some of the compounds, 200 mg was the maximum tolerated dose for rats.

[‡] The compounds used were supplied generously by Dr. Louis F. Fieser, Department of Chemistry, Harvard University, and by the Abbott Laboratories, North Chicago, Ill.

A Study of the Effect of Alloxan Diabetes on the Riboflavin Requirements of Young Rats.*

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Clinical as well as experimental evidence has at various times been interpreted to indicate that various members of the vitamins of the B complex may be useful in the control of diabetes mellitus or that the vitamin requirements of the diabetic animal are abnormally high. It would not be surprising if this were true in view of the well known functions of thiamine, riboflavin and niacin in the metabolism of carbohydrates.¹⁻³ Recent evidence implicates pantothenic acid⁴ and biotin⁵⁻⁸ in carbohydrate metabolism as well, and choline might be important indirectly in glucose metabolism through its effect upon liver function.

However, all of the clinical data⁹⁻¹⁷ may be

* Supported in part by grants-in-aid from the Nutrition Foundation, Inc., New York City, the Milbank Memorial Fund, New York City, and Swift & Company, Chicago, Ill.

¹ Baldwin, E., *Dynamic Aspects of Biochemistry*, The Macmillan Company, New York, 1947.

² Sumner, J. B., and Somers, G. F., *Chemistry and Methods of Enzymes*, New York, Academic Press, Inc., 1947.

³ Soskin, S., and Levine, R., *Carbohydrate Metabolism*, Chicago, University of Chicago Press, 1946.

⁴ Pilgrim, F. J., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 237.

⁵ Liehstein, H. C., and Umbreit, W. W., *J. Biol. Chem.*, 1947, **170**, 329.

⁶ Ochoa, S., Mehler, A., Blanchard, M. L., Jukes, T. H., Hoffmann, C. E., and Regan, M., *J. Biol. Chem.*, 1947, **170**, 413.

⁷ Summerson, W. H., Lee, J. M., and Pattridge, C. W. H., *Science*, 1944, **100**, 250.

⁸ Lardy, H. A., Potter, R. L., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **169**, 451.

⁹ Vorhaus, M. G., Williams, R. R., and Waterman, R. E., *J. A.M.A.*, 1935, **105**, 1580.

¹⁰ Seielounoff, F., *Rev. Med. Suisse Rom.*, 1940, **60**, 1243.

criticized on the basis that non-diabetic dietary controls were not available for study. The mere fact that the diabetic patient responds favorably to the administration of a vitamin does not necessarily indicate anything abnormal in the requirements or function of the vitamin but may simply reflect an inadequacy of the dietary intake as in any other individual. The experimental evidence is also unsatisfactory. Several authors^{15,18-22} have reported changes in the glucose tolerance, the blood sugar level, and the effectiveness of insulin when various vitamins were given to diabetic animals, but again the dietary control has been usually inadequate.

In a previous study from this laboratory²³ the thiamine requirement of the alloxan diabetic rat was found to be approximately the same as the non-diabetic controls and appeared to be related, as in the normal animal,

¹¹ Mosonyi, J., and Aszodi, Z., *Klin. Woch.*, 1938, **17**, 337.

¹² Sydenstricker, V. P., Geeslin, L. E., and Weaver, J. W., *J. A.M.A.*, 1939, **113**, 2137.

¹³ Neuwahl, F. J., *Lancet*, 1943, **2**, 348.

¹⁴ Gottlebe, P., *Zeit. f. Klin. Med.*, 1938, **133**, 739.

¹⁵ Labbe, M., Nepveux, F., and Gringoire, J. D., *Bull. Acad. de Med.*, 1933, **109**, 689.

¹⁶ Schroeder, H., *Z. f. Ges. Exp. Med.*, 1937, **101**, 373.

¹⁷ Stepp, W., Schroeder, H., and Altenburger, E., *Klin. Woch.*, 1935, **14**, 933.

¹⁸ Mosonyi, J., and Aszodi, Z., *Klin. Woch.*, 1938, **17**, 337.

¹⁹ Aszodi, Z., and Mosonyi, J., *Klin. Woch.*, 1937, **16**, 1214.

²⁰ Martin, R., *Z. Physiol. Chem.*, 1937, **248**, 242.

²¹ Tislowitz, R., *Klin. Woch.*, 1937, **16**, 226.

²² Magyar, I., *Zeit. f. Ges. Exp. Med.*, 1938, **104**, 495.

²³ Lowry, P. T., and Hegsted, D. M., *J. Lab. Clin. Med.*, 1945, **30**, 839.

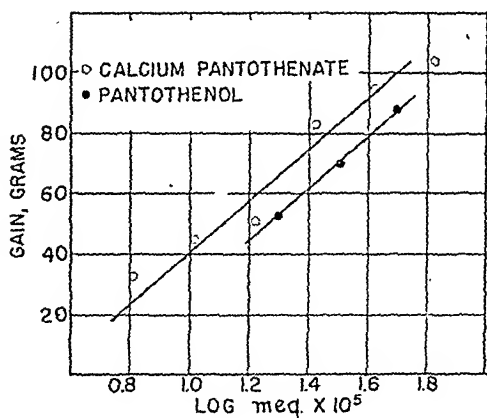


FIG. 1.

A comparison of the gain produced by pantothenic acid and pantothenol in chicks. The equation for the regression line for pantothenic acid is $Y = 86.80X - 59.17$, where Y is gain and X is log dose in milliequivalents $\times 10^5$.

day. The chicks were weighed every other day for 16 days.

The mean gain of each group of 6 chicks is shown in Fig. 1 where the dosage is given as the log of the milliequivalents administered in order to compare the two materials upon a reasonable basis. It is apparent that the log dose-response curve is essentially linear (calculated from the individual gain of each chick) and that the line representing the standard calcium pantothenate is parallel to that for the pantothenol. The potency ratio may therefore be calculated as with other

biological assays of this type.^{7,8} The distance between the lines in the X direction is 1.932, the antilog of which is 0.857. Thus pantothenol appears to yield 86% of the activity of pantothenic acid when compared upon an equivalent basis. Converting these figures into weights, pantothenol would appear to be about 91% as active as pantothenic acid, and equally as active as calcium pantothenate.

The relative accuracy of this assay compared to other biological assays of this type may be seen by comparing the standard deviation of the dose, s/b , with those tabulated by Bliss and Cattell,⁹ where s is the standard deviation about the line and b is the slope. For the calcium pantothenate line these values are 17.14 and 85.18, respectively, and the ratio is 0.201. This value is reasonably small as compared to other vitamin assays.

Summary. The chick assay for pantothenic acid, with the supplement administered daily by pipette, yields a reasonably straight log dose-response curve.

Pantothenol was found to have 86% of the activity of pantothenic acid compared milliequivalent for milliequivalent. Gram for gram it was 91% as active.

We are indebted to Merck & Co., Inc., Rahway, N. J., Corn Industries Research Foundation, New York City, and Sheffield Farms Company, Inc., New York City, for generous supplies of materials used in these studies.

The pantothenol was supplied by Hoffmann-La Roche, Inc., Nutley, N. J.

⁷ Coward, K. H., *The Biological Standardization of the Vitamins*, London, Bailliere, Tindall and Cox, 1938.

⁸ Bliss, C. I., *Ann. App. Biol.*, 1935, **22**, 139.

⁹ Bliss, C. I., and Cattell, M., *Ann. Rev. Physiol.*, 1943, **5**, 479.

TABLE II.
Comparison of the Response of Riboflavin Deficient Diabetic and Non-diabetic Animals to Various Levels of Riboflavin.

Riboflavin injected mcg/100 g body wt	Response period, days	Gain Coefficient		Wt Gain		Increase in food intake		Increase in water intake		Increase in riboflavin excretion	
		Diabetic	Control	Diabetic %	Control %	Diabetic %	Control %	Diabetic %	Control %	Diabetic mcg	Control mcg
160	1	3.46	2.78	19.4	23.9	205	131*	452	148*	20	17
	2-5					229	208	518	156	16	25
80	1	2.55	2.13	11.8	20.5*	81	83	366	159*	7	10
	2-5					33	84*	88	107	11	11
40	1	1.05	1.11	3.4	7.6	59	54	211	149	8	6

* Significant difference (probability 0.05 or less).

be seen, the diabetic and non-diabetic animals developed the deficiency in the same period of time as indicated by the 3 criteria used, i.e., loss of weight in 10 weeks, and the time required to show a loss in weight and reduction of food intake to 50% of the original intake.

The therapeutic responses were difficult to compare because of the fact that the diabetic rats, whether deficient or not, showed a reduced rate of gain in comparison to the non-diabetic animals. In order to obviate this difficulty to the best of our ability, we have compared the diabetic and non-diabetic rats not to each other but to themselves prior to the development of the deficiency, or as the percent response caused by the dose of riboflavin. In Table II the "gain coefficient" is the ratio of the daily gain during the first 5 days after the injection of riboflavin to the daily gain of the animal during the first 21 days of the study before riboflavin became a limiting factor. As may be seen, the diabetic animals showed only slightly larger relative gains in 2 of the test periods, but in no instance were they significantly different from the non-diabetic animals. The percentage increase in weight was not significantly different at the 160 and 40 mcg levels of riboflavin, but at 80 mcg the non-diabetic rats gave significantly greater gains.

Because of the immediate large increase in food and water intake following the administration of riboflavin to the diabetic animals, it appeared desirable to study the response during the first day and the subsequent 4 days as shown in Table II. The increase in food and water intake was greater in the diabetic group than in the non-diabetic group in several periods although in one period the control group showed a significantly greater food intake and somewhat larger water intake. It is doubtful if this is of real significance since as the deficiency develops and appetite fails, the urinary sugar of diabetic animals falls to low and nearly normal levels. When riboflavin is given the animals revert to their prior state in which sugar and water output are much above normal levels.

The increase in riboflavin excretion in the

TABLE I.
Comparison of the Rate of Development of Riboflavin Deficiency in Diabetic and Non-diabetic Rats.

Group	Loss of maximum wt in 10 wk, %	Time to reduce food intake to 50%, weeks	Time before wt loss began, days
Diabetic	20.8 \pm 1.5*	8.2 \pm 0.77*	33.3 \pm 3.74*
Non-diabetic	20.9 \pm 5.7	8.0 \pm 1.0	32.0 \pm 3.8

* Mean \pm standard deviation.

with the amount of glucose metabolised.

The present study represents an attempt to determine whether the riboflavin requirements of the rat are affected by alloxan diabetes. This appeared worthy of study since (a) the proportion of fat, protein and carbohydrate metabolised is changed during diabetes and this may affect the riboflavin requirements,²⁴⁻²⁶ (b) the development of cataracts is common in the diabetic rat²⁷⁻²⁸ and in riboflavin deficient rats in some laboratories,²⁹⁻³¹ and (c) the marked diuresis during the course of alloxan diabetes might influence the rate of excretion of the water soluble vitamins especially after the parenteral administration of relatively large amounts of these vitamins.

Experimental. Twenty-six young female rats weighing between 90 and 110 g were divided into two main groups. The first group of 14 animals was rendered severely diabetic by the intravenous administration of 50 mg alloxan monohydrate per kilo body weight. Five of these animals were used as diabetic controls and were given an ordinary purified

diet.³² The remainder of the diabetic rats received the same diet except that the riboflavin supplement was omitted. Twelve non-diabetic rats on the same riboflavin deficient diet were used as controls.

All rats were kept in individual cages and food and water given *ad libitum*. Daily records of weight, food, and water intake were kept and 3-day urine samples were collected through the whole experimental period and analyzed for glucose, using the method of Somogyi.³³

After the animals had become deficient, as indicated by their weight, they were injected with therapeutic doses of riboflavin. The growth response, change in food and water intake and urinary sugar, and excretion of riboflavin in the urine were studied. Urinary riboflavin was determined by a slight modification of the fluorometric method of Ferrebee³⁴ using the permanganate, sodium hydrosulfite procedure.

When the animals again began to lose weight after the therapeutic test, they were all injected with another dose of riboflavin. Three tests were thus obtained with each animal. The first dose of 160 mcg of riboflavin per 100 g body weight was given on the 70th day of the experiment. The second dose of 80 mcg per 100 g body weight was given 3 weeks later and the final dose of 40 mcg was given 16 days after the second. The urinary riboflavin during the 6 days prior to riboflavin injections ranged from 2.2 to 3.2 mcg per day per rat in both the diabetic and the non-diabetic rats.

Results. Table I indicates the data obtained during the depletion period. As may

²⁴ Unna, K., Singher, H. O., Kensler, C. J., Taylor, H. C., and Rhoads, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 254.

²⁵ Sarett, H. P., Klein, J. R., and Perlzweig, W. A., *J. Nutrition*, 1942, **24**, 295.

²⁶ Mannering, G. J., Lipton, M. A., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 100.

²⁷ Bailey, C. C., Bailey, O. T., and Leech, R. S., *New Eng. J. Med.*, 1944, **230**, 533.

²⁸ Foglia, V. G., and Cramer, F. K., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 218.

²⁹ O'Brien, C. S., *Arch. Ophthalmol.*, 1932, **8**, 880.

³⁰ Baum, H. M., Michaelree, J. F., and Brown, E. B., *Science*, 1942, **95**, 24.

³¹ Bessey, O. A., and Wolbach, S. B., *J. Exp. Med.*, 1939, **69**, 1.

³² Riggs, T. R., and Hegsted, D. M., *J. Biol. Chem.*, 1948, **172**, 539.

³³ Somogyi, M., *J. Lab. Clin. Med.*, 1941, **20**, 1220.

³⁴ Ferrebee, J. W., *J. Clin. Invest.*, 1940, **19**, 251.

following 50 sites: testis, tunic, cord extension, opposite testicle, cord or tunic, retro-peritoneal space, bladder, L. perirenal space, L. kidney, R. perirenal area, R. kidney, R. adrenal, L. adrenal, parietal peritoneum, serosa, omentum and ligaments, intestines, spleen, stomach, liver, pancreas, diaphragm, post-mediastinum, sup. med. and thymus, pleura, lungs, pericardium, heart, ant. cervical region, thyroid, parathyroids, muscle of tongue, muscle of mastication, mandible, teeth (mouth), eyes, pericranium, brain, hypophysis, nose and sinuses, post cerv. region, interscapular space, muscle of scapulae, muscle of thorax and abdo., skin of thorax and abdo., subcut. tissue thorax and abdo., muscle ant. portion of thigh, lower ends of femora, upper ends of tibiae, spinal canal. Paralyzed animals or animals with perforating eye metastases were killed and the day of death listed as the time of occurrence of the paralysis or perforation.

Results. Among the 42 New Zealand white rabbits inoculated intratesticularly there were at the end of a 60-day observation period, 37 with primary tumors which had not regressed (88%), 39 with metastases (93%), and 26 which had died from tumor metastases (mortality 62%). The metastatic foci averaged 13.4 (sites) per animal with metastases, 12.3 (sites) per animal with tumor at necropsy, and 11.4 (sites) per animal inoculated (Var. mean = 2.1). The primary tumors averaged by water displacement at necropsy 16.2 cc per animal with primary tumor, 16.2 cc per animal with tumor, and 15.0 cc per animal inoculated. The metastatic tumor averaged 105.6 cc by water displacement at necropsy per animal with metastases, 97.4 cc per animal with tumor and 90.5 cc per animal inoculated.

Among the 66 New Zealand white rabbits inoculated subcutaneously there were at the end of the 60-day observation period 31 with primary tumor which had not regressed (47%), 33 with metastases (50%), and 9 which had died from the effects of tumor metastases (mortality 13%). The metastatic foci averaged 8.2 (sites) per animal with metastases, 6.4 (sites) per animal with tumor, and 3.2 (sites) per animal inoculated (Var. mean = 0.59). The primary tumors

averaged by water displacement at necropsy 48.6 cc per animal with primary tumor, 45.7 cc per animal with tumor at necropsy, and 22.8 cc per animal inoculated. The metastatic tumor averaged by water displacement at necropsy 33.2 cc per animal with metastases, 26.1 cc per animal with tumor, and 13.1 cc per animal inoculated.

Each of the above listed differences between the course of tumor after transplantation by the subcutaneous and by the intratesticular routes was statistically significant.³ For instance, the difference in the mortality of 13% and 62% was significant ($X^2 = 25.1$, $N = 1$, $P = 0.0001$ —), and in the number of metastatic foci per animal inoculated of 11.4 and 3.2 (diff. = 8.2 ± 1.64 ; $t = 5.0$; $P = 0.0001$ —).

Generally, the two best criteria for this neoplasm are the mortality from the tumor in

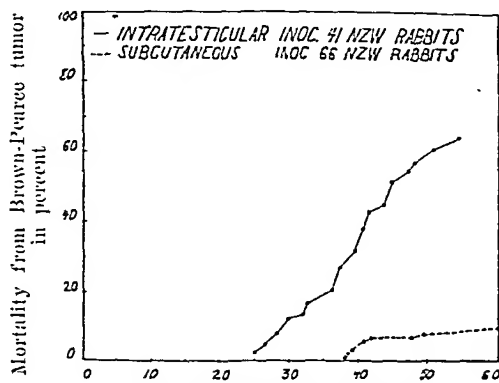


FIG. 1.

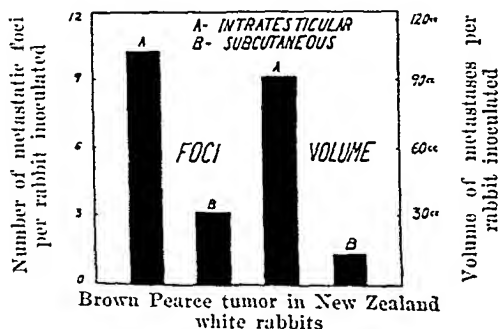


FIG. 2.

³ Fisher, R. A., Statistical Methods for Research Workers, 1938, 7 ed., Oliver and Boyd, London.

urine above the level prior to riboflavin injection was in no instance significantly different in the diabetic and non-diabetic groups. It seems apparent that the large urinary volume in the diabetic animals do not "wash out" riboflavin to a significant degree, if at all. Attempts to find a significant correlation between the amount of riboflavin retained and food intake, water intake, gain after dosage with riboflavin, or loss of body weight during the depletion period were unsuccessful.

All diabetic animals including those which received riboflavin developed cataracts after 55 to 65 days while none of the non-diabetic riboflavin-deficient animals developed cataracts during the 110 days of the study.

Conclusion. Studies upon the rate of development of riboflavin deficiency and the response to therapeutic doses of riboflavin, after the development of the deficiency syndrome, failed to show any significant difference in the riboflavin requirement of the alloxan diabetic and the non-diabetic rat. Similarly, riboflavin deficiency was apparently without effect upon the development of cataracts in the diabetic animals.

We are indebted to Merek & Co., Rahway, N. J., Corn Industries Research Foundation, New York City, and Sheffield Farms Company, Inc., New York City, for generous supplies of materials used in these studies.

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Intratesticular Versus Subcutaneous Transplantation of Brown-Pearce Tumor in New Zealand Whites.

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Published data¹ on the susceptibility of various rabbit breeds to the Brown-Pearce tumor failed to include the breed most readily procurable on the open market, namely, the New Zealand White. During the past several years 108 New Zealand white male rabbits have been used as controls or stock animals in experiments with the Brown-Pearce tumor, 42 being inoculated intratesticularly and 66 subcutaneously.

Materials and methods. The 66 animals inoculated subcutaneously belonged to 16 different groups distributed throughout the year as follows: January 24 (1), March 6 (2), March 20 (3), May 22 (5), June 23 (8), June 24 (8), July 24 (9), August 11 (4), September 6 (10), October 9 (8), October 31 (3), November 15 (2), December 13 (1), and December 30 (2).

The 42 animals inoculated intratesticularly belonged to 16 groups distributed as follows: January 24 (1), February 28 (6), June 9 (1), June 23 (1), July 17 (1), August 3 (4), August 30 (2), September 16 (1), September 24 (3), October 16 (2), November 9 (3), November 20 (1), November 22 (3).

The 7 animals comprising the two groups inoculated subcutaneously August 11 and October 31 were 3 months of age, others inoculated subcutaneously and all of those inoculated intratesticularly were animals of 4-12 months of age. Animals inoculated subcutaneously and those inoculated intratesticularly were obtained in almost equal proportions from the same breeders or dealers in Alabama, Louisiana, Illinois, Virginia and Pennsylvania.

As in the earlier studies,² a metastatic focus was defined as the presence of one or more tumor nodules at necropsy in any of the

* The work was aided by a grant through the Committee on Growth, of The National Research Council, Acting for The American Cancer Society.

¹ Casey, Albert E., *Am. J. Cancer*, 1937, 21, 446.

² Casey, Albert E., *Am. J. Cancer*, 1931, 21, 760; 1936, 26, 276.

following 50 sites: testis, tunic, cord extension, opposite testicle, cord or tunic, retro-peritoneal space, bladder, L. perirenal space, L. kidney, R. perirenal area, R. kidney, R. adrenal, L. adrenal, parietal peritoneum, serosa, omentum and ligaments, intestines, spleen, stomach, liver, pancreas, diaphragm, post-mediastinum, sup. med. and thymus, pleura, lungs, pericardium, heart, ant. cervical region, thyroid, parathyroids, muscle of tongue, muscle of mastication, mandible, teeth (mouth), eyes, pericranium, brain, hypophysis, nose and sinuses, post cerv. region, interscapular space, muscle of scapulae, muscle of thorax and abdo., skin of thorax and abdo., subcut. tissue thorax and abdo., muscle ant. portion of thigh, lower ends of femora, upper ends of tibiae, spinal canal. Paralyzed animals or animals with perforating eye metastases were killed and the day of death listed as the time of occurrence of the paralysis or perforation.

Results. Among the 42 New Zealand white rabbits inoculated intratesticularly there were at the end of a 60-day observation period, 37 with primary tumors which had not regressed (88%), 39 with metastases (93%), and 26 which had died from tumor metastases (mortality 62%). The metastatic foci averaged 13.4 (sites) per animal with metastases, 12.3 (sites) per animal with tumor at necropsy, and 11.4 (sites) per animal inoculated (Var. mean = 2.1). The primary tumors averaged by water displacement at necropsy 16.2 cc per animal with primary tumor, 16.2 cc per animal with tumor, and 15.0 cc per animal inoculated. The metastatic tumor averaged 105.6 cc by water displacement at necropsy per animal with metastases, 97.4 cc per animal with tumor and 90.5 cc per animal inoculated.

Among the 66 New Zealand white rabbits inoculated subcutaneously there were at the end of the 60-day observation period 31 with primary tumor which had not regressed (47%), 33 with metastases (50%), and 9 which had died from the effects of tumor metastases (mortality 13%). The metastatic foci averaged 8.2 (sites) per animal with metastases, 6.4 (sites) per animal with tumor, and 3.2 (sites) per animal inoculated (Var. mean = 0.59). The primary tumors

averaged by water displacement at necropsy 48.6 cc per animal with primary tumor, 45.7 cc per animal with tumor at necropsy, and 22.8 cc per animal inoculated. The metastatic tumor averaged by water displacement at necropsy 33.2 cc per animal with metastases, 26.1 cc per animal with tumor, and 13.1 cc per animal inoculated.

Each of the above listed differences between the course of tumor after transplantation by the subcutaneous and by the intratesticular routes was statistically significant.³ For instance, the difference in the mortality of 13% and 62% was significant ($\chi^2 = 25.1$, $N = 1$, $P = 0.0001$ —), and in the number of metastatic foci per animal inoculated of 11.4 and 3.2 (diff. = 8.2 ± 1.64 ; $t = 5.0$; $P = 0.0001$ —).

Generally, the two best criteria for this neoplasm are the mortality from the tumor in

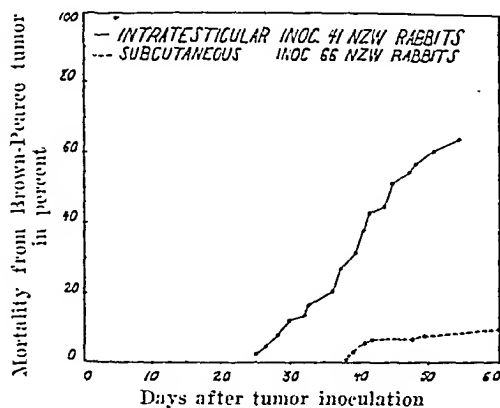


FIG. 1.

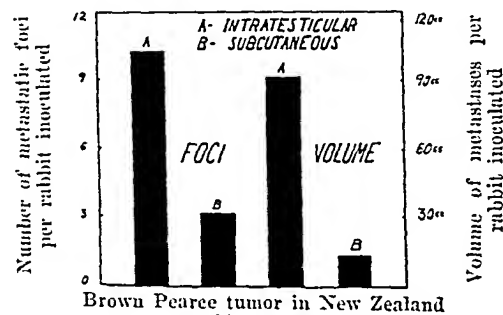


FIG. 2.

³ Fisher, R. A., Statistical Methods for Research Workers, 1938, 7 ed., Oliver and Boyd, London.

days after inoculation, and the number of metastatic foci determined by careful necropsy and confirmed by microscopic sections.² These values are shown in Fig. 1 and 2.

Discussion. The total primary and metastatic tumor per animal with metastases was 82 cc per animal inoculated subcutaneously, and 122 cc per animal inoculated intratesticularly; or an average per metastatic focus of 8.9 cc (for the 9.2 primary and metastatic foci) in the former, and 8.4 cc (for the 14.4 primary and metastatic foci) in the latter. It is obvious that the average rate of growth of the tumor was approximately the same in any given site. Under the skin the primary tumor grew larger as there was abundant area to expand. In the testis the growth was confined and spread up the cords to the perirenal areas, retroperitoneal space and peritoneal cavity by simple extension. It has been proven that the pattern of local spread is determined by the site of injection of this tumor;⁴ also the pattern of the distant metastases in the Brown-Pearce and in other tumors is not altered or affected by the site origin of the primary tumor⁴ nor by the breed of rabbit employed.⁵

The differences between the subcutaneous and the intratesticular routes were not due to the season of the year in which the animals were inoculated.⁶ The rabbits inoculated during October, November, December, January, February and March averaged 12.2 metastatic foci among the 16 rabbits transplanted intratesticularly and 4.68 foci among the 22 transplanted subcutaneously; the rabbits inoculated during May, June, July, August and September averaged 10.9 metastatic foci for the 21 rabbits transplanted intratesticularly

and 2.48 metastatic foci for the 44 rabbits transplanted subcutaneously. Nor were the differences due to age since the elimination of the 7 animals 3 months of age did not appreciably affect the mean number of metastatic foci or the mortality.

The possibility was considered that the New Zealand Whites obtained from such widely separated areas as Pennsylvania, Illinois, and Alabama might represent stock variations. Such stock variations undoubtedly did occur but the animals injected by the two routes were obtained in roughly proportioned numbers from the various areas. Furthermore, Havana, Himalayana, and Flemish stock obtained also from dealers in the various states have given almost the same data on mortality and metastatic foci as was obtained from inbred lines some 15 years ago.¹ It still remains true that no Havana rabbit has died from the Brown-Pearce tumor, although metastases have occasionally been noted. Approximately 30% of all animals (including all breeds and hybrids) inoculated subcutaneously have died from metastases within a 2-3 months observation period. This may explain a variety of results reported by various authors trying to produce immunity by the subcutaneous route. Nozu was the first to observe metastases of the Brown-Pearce tumor by intracutaneous inoculation alone⁷ and Casey induced metastases by the intracutaneous route using the Brown-Pearce XYZ factor.⁸

Brown and Pearce noted the marked tendency to metastasis following intratesticular inoculation of the rabbit tumor and an absence of metastasis following intra- and subcutaneous inoculation.⁹ Pearce and Brown were the first to point out that rabbits inoculated with the Brown-Pearce tumor intra- or subcutaneously or intramuscularly "developed an immunity which was sufficient to protect them from subsequent inoculation by the same route or from inoculation made into the

¹ Casey, Albert E., and Pearson, Bjarne, *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 234.

² Casey, Albert E., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 223.

³ Brown, Wade H., Pearce, Louise, and Van Allen, C. M., *Proc. Soc. Exp. Biol. and Med.*, 1924, **21**, 371; Pearce, Louise, and Van Allen, C. M., *Proc. Soc. Exp. Biol. and Med.*, 1925, **22**, 448; *J. Exp. Med.*, 1927, **45**, 483; Pearce, Louise, and Brown, Wade H., *J. Exp. Med.*, 1927, **45**, 727.

⁷ Nozu, Y., *Acta dermat.*, 1933, **20**, 66.

⁸ Casey, Albert E., *Am. J. Cancer*, 1934, **21**, 776.

⁹ Brown, Wade H., and Pearce, Louise, *J. Exp. Med.*, 1923, **37**, 799.

testicles."¹⁰ Their observations were confirmed by Casey⁸ and by Besredka, Mazat, Besnard, Gross, Bardach, Laval,¹¹ Saphir, Appel, Strauss,¹² Cheever, and Janeway.¹³ Besredka and Gross reported that this immunity could be produced only by the tumor cells and that it could not be induced by the intracutaneous inoculation of normal tissues.¹¹ In Fig. 1 it will be seen that the New Zealand white rabbit shows no evidence of developing at about 40 days an immunity or resistance following intratesticular inoculation as is true

for this same breed following subcutaneous inoculation or for common hybrids by intratesticular inoculation as reported by Brown and Pearce^{9,10} and by Malluche.¹⁴

Summary and conclusions. Standard values were compiled for the Brown-Pearce tumor in New Zealand white male rabbits, 42 being inoculated intratesticularly and 66 subcutaneously. The breed was highly resistant to subcutaneous inoculation, (mortality, 13.6%; metastatic foci, 3.2; metastatic tumor 13.1 cc per animal inoculated) and susceptible to intratesticular inoculation (mortality 62.7%, metastatic foci 11.4 and metastatic tumor 90.5 cc per animal inoculated). Between 40 and 60 days after intratesticular inoculation the mortality curve continued to rise at about the same rate as between 20 and 40 days. This was in contrast to the immune or resistance reaction which seemed to set in between 40 and 60 days following subcutaneous inoculation of this breed, or following intratesticular inoculation of most other breeds.

¹⁰ Pearce, Louise, and Brown, Wade H., *J. Exp. Med.*, 1923, **37**, 811.

¹¹ Besredka, A., Mazat, I., and Besnard, P., *Comp. rend. Acad. d. sc.*, 1935, **201**, 170; Gross, L., *Am. J. Cancer*, 1937, **31**, 609; Besredka, A., and Bardach, M., *Comp. rend. Acad. d. sc.*, 1936, **203**, 2193; Besredka, A., *Cancer Bruxelles*, 1935, **12**, 115; Besredka, A., Mazat, I., Laval, P., and Besnard, P., *Ann. Inst. Pasteur*, 1936, **36**, 125; Besredka, A., and Gross, L., *Ann. Inst. Pasteur*, 1936, **37**, 342; 1938, **60**, 5, 465; 1939, **62**, 253.

¹² Saphir, O., and Appel, M., *Am. J. Cancer*, 1940, **38**, 55; Saphir, O., Appel, M., and Strauss, A. A., *Cancer Research*, 1941, **1**, 545.

¹³ Cheever, F. S., and Janeway, C. A., *Cancer Research*, 1941, **1**, 23.

¹⁴ Malluche, H., *Beitr. Z. klin. Chir.*, 1938, **167**, 481.

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Selective Blocking of Host Resistance to Malignant Neoplasm (Brown-Pearce Tumor in New Zealand White Rabbits).

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In the preceding paper¹ it was reported that the New Zealand White rabbit is highly susceptible following intratesticular and highly resistant following subcutaneous transplantation of the Brown-Pearce tumor. In the mortality curve an immune or resistance

reaction could be detected at about 40-60 days following subcutaneous inoculation but little or none following intratesticular inoculation of the tumor. The present experiments were designed to test the extent to which the XYZ factor² might overcome this immune or resistance reaction of the New Zealand white to this tumor following subcutaneous inoculation.

* The work was aided by a grant from the Committee on Growth of the National Research Council, acting for the American Cancer Society.

¹ Casey, Albert E., Meyers, L., and Drysdale, George R., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 576.

² Casey, Albert E., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 816; *Am. J. Cancer*, 1934, **21**, 760; 1936, **26**, 276; 1939, **35**, 354.

TABLE I.
Preparation of and Injection Interval for XYZ Factor from Brown-Pearce Tumor (134 New Zealand Whites).

Exp.	Preparation			Injection XYZ			Animals inoculated Brown-Pearce tumor	
	Temp.	Storage, days	Covering	Site	Dosage, g	Interval, days	Exp.	Controls
1. a.	24°F	15	Paraffin	S.C.	0.0001	19	4 S.C.	4 S.C.
b.	24 "	15	"	"	0.01	19	5 "	5 "
c.	24 "	15	"	"	1.0	19	7 "	7 "
2. a.	34 "	25	Glycerin	"	0.0001	16	2 "	2 "
b.	34 "	25	"	"	0.01	16	3 "	3 "
c.	34 "	25	"	"	1.0	16	3 "	5 "
3.	-22°C	30?	None	"	0.02	15	9 "	8 "
4.	-22 "	30?	"	"	0.02	16	5 "	8 "
5. a.	0°F	244	Glycerin	I.T.	0.1	11	3 "	} 4 "
b.	0 "	244	"	"	(0.1) ²	11	4 "	
6. a.	0 "	296	"	"	0.1	11	4 "	} 4 "
b.	0 "	296	"	"	(0.1) ²	11	4 "	
7.	0 "	51	None	S.C.	0.1	21	1 "	2 "
Subtotal (Subcutaneous)							54	52
1.	18 "	14	Paraffin	I.T.	0.1	14	5 I.T.	8 I.T.
2.	18 "	10	"	"	0.1	10	6 "	9 "
Subtotal (Intratesticular)							11	17
Total subcutaneous and intratesticular							65	69

Material and Methods. There were 13 experiments involving 106 New Zealand white rabbits inoculated subcutaneously and several experiments involving 28 New Zealand white rabbits inoculated intratesticularly. In the former 52 controls and 54 experimental animals were inoculated subcutaneously with 0.2 to 0.3 cc of an emulsion of tumor tissue, and in the latter 17 controls (including 6 stock animals) and 11 experimental animals inoculated intratesticularly with a similar dosage.

The XYZ material consisted of aseptically removed Brown-Pearce tumor (from 7 different animals) covered with paraffin, or layered with 50% glycerine in normal saline, or even left in a sterile container uncovered. The tumor tissue was refrigerated at 24°-34° F for 15-25 days (always covered with paraffin or glycerin at these temperatures), or at 0° F or lower for periods up to 300 days (Table I). Immediately after removal from the ice chamber amounts of the refrigerated tumor tissue (in the equivalent of 0.0001 to 1.0 g)

were emulsified in normal saline and given as a single injection (see exceptions below) subcutaneously or intratesticularly 11-21 days prior to subcutaneous or intratesticular transplantation of the Brown-Pearce tumor (Table I). One group (in Experiments 5 and 6) was injected twice with XYZ material after a week's interval. The variations in the dosage of the frozen material seemed not to be significant.³ About half of the animals were obtained directly from the breeder and it was often possible to divide them so that one litter mate would be in the experimental and one in the control group. In any event the controls and experimental animals were matched according to breed and weight before beginning the experiments with equal numbers in each. About 10% of both experimental and control animals died from intercurrent disease, and were eliminated, thus accounting for the unequal groups (Table I). The ex-

³ Casey, Albert E., *Cancer Research*, 1941, 1, 134.

TABLE II.

Brown-Pearce Tumor in New Zealand White Rabbits as Influenced by the Prior Injection of the Brown-Pearce XYZ Factor (Subcutaneous Transplantation of Tumor).

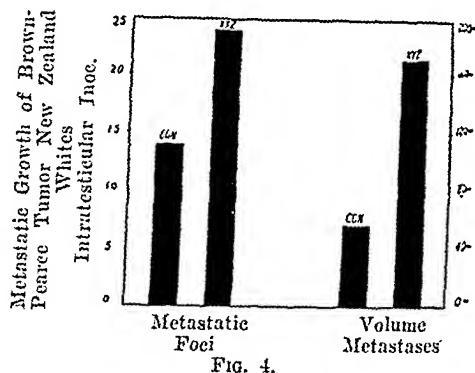
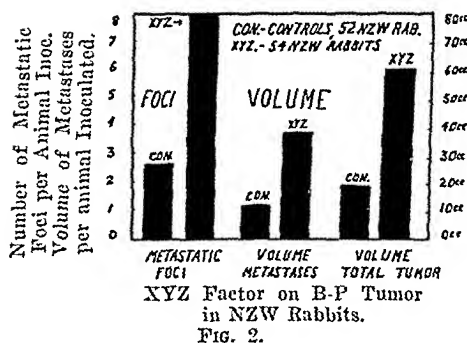
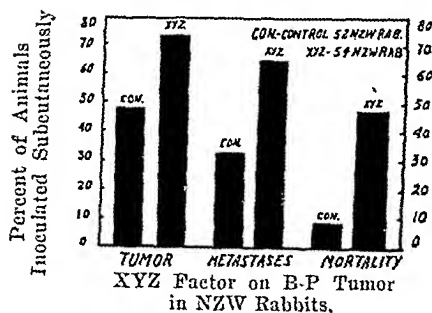
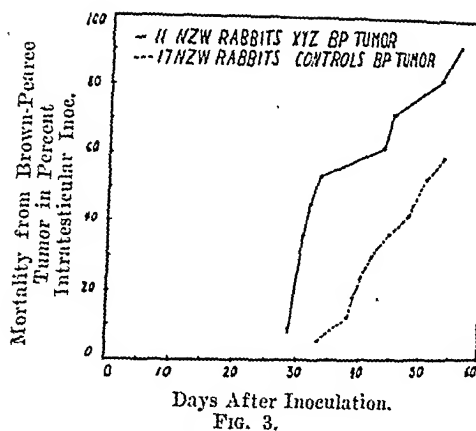
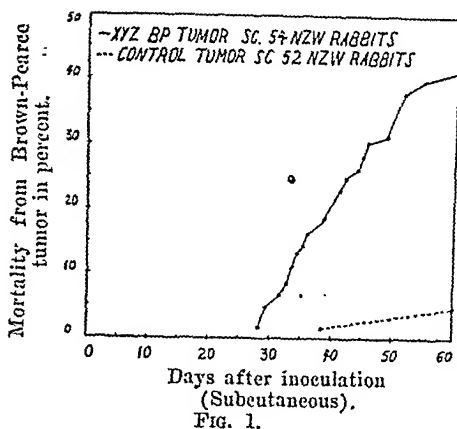
	Non-regressed tumor, necropsy		Metastases		Died from metastases		Total
	Animals	%	Animals	%	Animals	%	animals
XYZ	39	(72)	35	(65)	25	(46)	54
Controls	24	(46)	17	(33)	4	(8)	52
Chi square	9.7		10.9		19.9		
Prob.	0.01 sig.		0.01 sig.		0.01 sig.		
Metastatic Foci (or Sites) per Animal.							
	Inoculated		With tumor		With metastases		
	Mean	Variance	Mean	Variance	Mean	Variance	
XYZ	8.0	1.6	11.1	2.3	12.4	2.4	
Controls	2.6	0.6	5.6	2.4	7.9	3.7	
Diff.	5.4 ± 1.5		5.5 ± 2.2		4.5 ± 2.5		
Prob.	0.01 sig.		0.01 sig.		0.07		
Volume of the Metastases per Animal.							
	Inoculated		With tumor		With metastases		
	Mean, cc	Variance	Mean, cc	Variance	Mean, cc	Variance	
XYZ	38.7	55.9	53.6	88.4	59.8	99.5	
Controls	11.2	25.1	24.3	106.8	34.4	195.2	
Diff.	27.5 ± 9.0 cc		29.3 ± 13.9 cc		25.4 ± 17.1 cc		
Prob.	0.01 sig.		0.03 prob. sig.		0.15		
Volume of Primary and Metastatic Tumor per Animal.							
	Inoculated		With tumor				
	Mean, cc	Variance	Mean, cc	Variance			
XYZ	60.4	135.9	83.6	214.4			
Controls	17.8	53.7	38.5	223.3			
Diff.	42.6 ± 13.8 cc		45.1 ± 20.9 cc				
Prob.	0.01 sig.		0.03 prob. sig.				

TABLE III.

Brown-Pearce Tumor in New Zealand White Rabbits as Influenced by the Prior Injection of the Brown-Pearce XYZ Factor (Intratesticular Transplantation of Tumor).

	Non-regressed tumor, necropsy		Incidence of metastases		Died from metastases		Total inoculated	
	XYZ	Controls	XYZ	Controls	XYZ	Controls	XYZ	Controls
Animals	11	16	11	16	11	11	11	17
Percent	(100)	(94)	(100)	(94)	(100)	(65)	(100)	(100)
	Metastatic foci (sites)		Volume of metastases, cc		Total tumor primary and metastatic, cc			
Per animal inoculated	23.5	13.7	170.3	53.8	189.4	73.3	11	17
	diff. 9.8 ± 3.0		diff. 116.5 ± 22.1		diff. 116.1 ± 19.5			
	t = 3.2		t = 5		t = 6.0			
	P = 0.01—sig.		P = 0.01—sig.		P = 0.01—sig.			
Per animal with tumor	23.5	14.6	170.3	57.3	189.4	77.9	11	16
	diff. 8.9 ± 2.3		diff. 113.0 ± 23.6		diff. 111.5 ± 25.1			
	t = 3.87		t = 4.8		t = 4.4			
	P = 0.01—sig.		P = 0.01—sig.		P = 0.01—sig.			

perimental and control animals of two groups were only 3 months of age at the time of inoculation; the others were 4-12 months of age. All except one were males (the exception was among the 54 XYZ animals inoculated subcutaneously).



Both the control and experimental animals in each group were transplanted with the same emulsion and dosage of Brown-Pearce tumor. The animals were observed at weekly intervals and surviving animals sacrificed at 50 to 90 days. Careful necropsies according to a plan described in the preceding paper, were checked in each instance by the preparation of slides for microscopic examination. The size of the primary and metastatic tumor was measured at necropsy by water displacement.

Results. Among the 54 XYZ animals in-

oculated subcutaneously and the 11 XYZ animals inoculated intratesticularly with the Brown-Pearce tumor there was a greater incidence of and larger primary tumors, a greater incidence and volume of, and more numerous metastases and a greater mortality from the tumor in a shorter interval after inoculation than among their respective controls (52 controls were inoculated subcutaneously and 17 intratesticularly. Tables II and III, Fig. 1, 2, 3 and 4). Each of the differences was statistically significant for the groups inoculated subcutaneously and for those inoculated intratesticularly with two exceptions. The exceptions relate to the intratesticular inoculation in that 16 of the 17 controls (94%) had non-regressed primary tumor and metastases at necropsy, as compared with 11 (100%) among the XYZ injected animals. This afforded no opportunity to show a statistically significant XYZ effect.

Discussion. The production of a state of

hypersusceptibility to tumor transplantation has been, during the past fifty years, fraught with great difficulties. The only recorded instance where published observations could be confirmed by other workers was the demonstration by Haaland,⁴ by Leitch,⁵ and by Casey⁶ that such a state could be produced in the mouse using Bashford carcinoma 63. Sterile Bashford carcinoma 63 tissue was frozen and preserved anaerobically in the frozen state for 10-21 days. An emulsion of the frozen tumor tissue, no longer capable of growth, was injected subcutaneously into mice 10-21 days before subcutaneous transplantation of the same tumor. The animals so injected grew larger primary tumors than their controls not so injected. Some 200 control and 200 experimental animals were employed by the three authors. No metastases were observed and no further observations have been recorded.

Casey² was the first to demonstrate that similarly frozen and anaerobically preserved Brown-Pearce tumor tissue, no longer capable of growth, would when injected (1.0 to 0.0001 g) prior to tumor transplantation result not only in a greater incidence of and larger primary tumors but also in a greater incidence, number and volume of the metastases and in a greater mortality from the tumor in a shorter interval after transplantation than in control animals not so injected. The factor responsible was thermolabile⁷ probably specific for the Brown-Pearce tumor (homologous),^{8,9} filtrable through a Berkefeld "V" filter, differed from the Duran-Reynals spreading factor and effective even when injected into animals carrying the tumor. Because the phenomenon had no known counterpart in biology and the nature of the factor was unknown, the name

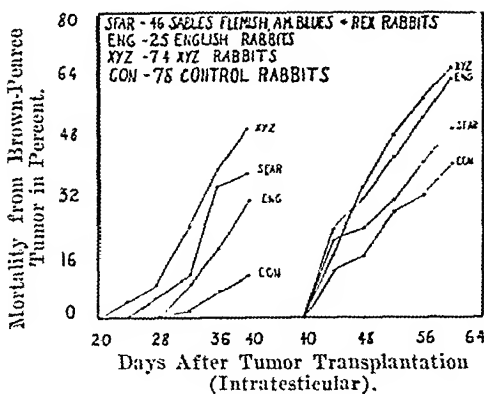


FIG. 5.

"XYZ" was given. Our total experience to date with the unaltered Brown-Pearce XYZ factor (including the present data) consists of 14 experiments involving 74 XYZ and 78 control animals in which the material was injected 10-21 days prior to intratesticular inoculation of this tumor, and 11 experiments involving 84 XYZ and 79 control animals inoculated subcutaneously. In each one of the 25 experiments during a 17 year period was the XYZ effect observed. The mortality curves for the groups inoculated intratesticularly were calculated first for the period up to 40 days and secondly for the period of 40-61 days after tumor transplantation and are presented (Fig. 5). The separation of mortality into the two periods was affected because the host factors influencing the first 40 days seem to differ from the host factors influencing the course of the tumor 40 to 60 days.^{10,12} The animal breeds used varied widely but were always matched between controls and experimental animals; the consistency of the results bears this out.

Animals of average resistance to this neo-

⁴ Haaland, M., *Proc. Roy. Soc. London*, 1910, **82**, 293; *Lancet*, 1910, **1**, 787.

⁵ Leitch, A., *Lancet*, 1910, **1**, 991.

⁶ Casey, Albert E., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 674.

⁷ Casey, Albert E., and Moragues-Gonzales, Vincent, *Am. J. Cancer*, 1940, **38**, 59.

⁸ Casey, Albert E., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 1025; 1934, **31**, 663; 1939, **42**, 731.

⁹ Casey, Albert E., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 111.

¹⁰ Malluche, H., *Beitr. Z. Klin. Chir.*, 1938, **167**, 481.

¹¹ Kidd, John G., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 292; *J. Exp. Med.*, 1946, **83**, 227.

¹² Casey, Albert E., and Drysdale, George R., *Cancer Research*, 1947, **7**, 728.

¹³ Casey, Albert E., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 666.

plasm (controls) became somewhat more susceptible than the animals 'of the most susceptible breeds (English, Flemish, Sable, American Blue and Rex) (Fig. 5). The factor has not been obtained (using identical techniques) from other neoplasms of the mouse or rabbit, nor from the rabbit spleen, skeletal muscle, or rabbit testis. The effect of the material has not in our hands resembled anaphylaxis or sensitization of the usual sort. Necroses do not appear at the site of repeated injections and animals injected weekly for almost a year were still highly susceptible and none died because of the injections. Histologically there seems less necrosis in the tumor tissue of XYZ animals and more cells seem alive. The prolonged storage in the frozen state seems to inactivate or mask such inhibitory factors as may be present in the fresh tumor tissue or the surrounding intercellular fluids.¹²

Kidd,¹¹ using our technique, froze Brown-Pearce tumor tissue and kept it frozen for 1-2 months at -22° C until ready for use. He then injected a saline emulsion of the frozen and non-viable tumor tissue, in the manner of our experiments, 7-20 days prior to intramuscular transplantation of the tumor. Among the 16 "blue cross" control rabbits was one animal at 30-35 days with progressively enlarging primary tumor (6%), none had metastases (0%) and none died from the tumor (0%). Among the 53 "blue cross" XYZ animals (called "immunized animals" by Kidd) there were 18 at 30-35 days with progressively enlarging primary tumors (34%), at least 10 had metastases (19%) and 10 died from metastases (19%). Evaluation of his work is difficult since his experiments were terminated at 30-35 instead of at the usual 60 days, control animals were few and autopsies were incomplete in that no enumerations of the incidence, number and volume of the metastases was recorded. Nevertheless his "immunized" (XYZ injected) animals had significantly more enlarging tumors ($X^2=4.7$, $P=0.035$), and among those with tumors a greater mortality from metastases ($X^2=5.6$, $n=1$, $P=0.02$) than their respective controls. The term, "immunized," used by Kidd with respect to the

XYZ injected animals is, we believe, ill considered.

Several years ago Dr. Kidd kindly sent us 2 samples of frozen Brown-Pearce tumor tissue tested by him for antigen. This non-viable tumor tissue was injected by us into New Zealand white rabbits 15-16 days prior to subcutaneous transplantation of the tumor. The animals comprise Groups 3 and 4 in the present experiments (Table I). Among the 16 controls 3 had non-regressed tumors at necropsy (19%), 2 had metastases (13%), and none had died (0%); the metastatic foci averaged 1.3 and the total tumor 3.6 cc per animal inoculated. Among the 14 XYZ animals 8 had non-regressed tumor at necropsy (57%), 5 had metastases (36%), 4 died from metastases (29%); the metastatic foci averaged 5.8 and the total tumor 26.4 cc per animal inoculated. Combining Dr. Kidd's animals with enlarging primary tumors at 30-35 days with our animals having non-regressed primary tumor at 60 days there were 26 such animals among the 67 "immunized" (XYZ injected), as compared with 4 among the 32 controls ($X^2=7.1$, $n=1$, $P=0.01$ -significant). Similarly the deaths from tumor were 14 among the 67 "immunized" (XYZ) and none among the 32 controls ($X^2=8.8$, $n=1$, $P=0.01$ -significant).

Kidd like ourselves encountered the XYZ phenomenon while trying to immunize with frozen tumor tissue. An antibody response to the frozen tumor tissue was obtained by him in 22 of the 53 "blue cross" rabbits in the form of complement fixing antibodies with titers of 1-2 to 1-64; and each of the 13 rabbits which failed to grow a primary tumor was in this group. Such an antibody response was absent or insignificant among rabbits of other breeds and mixtures when tested by Cheever,¹⁴ by Jacobs and Houghton¹⁵ and by Kidd.¹¹ The antibody response seems largely limited to the "blue cross" rabbit, which is a Lilac cross developed by Dr. Wade H. Brown for stock use at the Rocke-

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¹⁵ Jacobs, J. J., and Houghton, J. D., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 88.

feller Institute. The Lilac and its close relative, the Havana, have the highest natural resistance of any breeds tested against the Brown-Pearce tumor. No Havana rabbit has ever died from the tumor. Dmochowski¹⁶ and Jacobs and Houghton¹⁵ comment that it is "necessary to exclude the obvious likelihood that the antigenic substance is part of the cellular elements of the tumor" which result "in the formation of antibodies" when injected into a genetically different host. The antibodies described by Kidd seem to bear no relation to the growth of the Brown-Pearce tumor in animals successfully transplanted (in direct contrast to the XYZ effect). Among the 9 animals with antibodies and successfully transplanted 5 had enlarging primary tumors at the termination of the experiments (55%) and there were 2 deaths from metastases (22%), as compared with 13 enlarging primary tumors (42%) and 8 deaths from metastases (26%) among the 31 animals without antibodies and successfully transplanted. The differences were not significant.

Known mammalian viruses¹⁷ are thermolabile (55-60°C) yet Kidd has applied the term "virus" to the thermostable (65°C) antigen in the cells of the Brown-Pearce tumor causing antibody response in the Lilac cross or Blue cross rabbit. He disregards the specific filtrable thermolabile (56°C) XYZ factor, uniformly present in the frozen Brown-Pearce tumor tissue, and capable of abrogating or completely blocking host resistance to this neoplasm. Its effect is not limited to certain strains of rabbits. The only biologically comparable filtrable thermolabile factor in mammalian tumors is the milk factor, discovered more recently by Bittner.¹⁸ This agent is also specific, thermolabile

(56°C), filtrable, and also does not immunize but renders even resistant strains of mice highly susceptible to the development of a specific neoplasm. It also causes no necrosis, or obvious reaction in the host.

Summary and Conclusions. 1. Of 134 New Zealand White rabbits in 15 experiments 65 were given an injection of 1.0 to 0.0001 g of Brown-Pearce tumor tissue (no longer viable) which had been kept frozen anaerobically for 10-296 days. Viable Brown-Pearce tumor tissue was transplanted 10-21 days later into the testes of 54 and beneath the skin in 11 of the 65 animals; also inoculated at the same time were the 69 controls, 52 subcutaneously and 17 intratesticularly.

2. The experimental animals had a significantly greater incidence of and larger primary tumors, a greater incidence and volume of, and more numerous metastases and a greater mortality from the tumor in a shorter interval after inoculation than their respective controls, by both the intratesticular and subcutaneous routes.

3. This effect (XYZ factor) was not confined to the New Zealand White breed as common rabbit hybrids of average resistance to the Brown-Pearce tumor could be rendered more susceptible than even the most susceptible breeds such as the English, Sable, Flemish and Rex. Even the relatively resistant blue-cross or Lilac cross rabbit became more susceptible upon injection of the frozen tumor tissue.

4. The mechanism of the XYZ phenomenon seems to be influenced by the inactivation or masking by prolonged freezing of inhibitory factors present in the fresh tumor. The frozen material seems to act by blocking host resistance to a specific tumor.

¹⁶ Dmochowski, L., *Compt. rendue de la Soc. de Biol.*, 1938, 349.

¹⁷ Seiffert, Gustav, *Virus Diseases in Man, Animal and Plant*, Philosophical Libr., New York, 1944, 49.

¹⁸ Bittner, J. J., *Science*, 1936, **84**, 162; Anderson, H. B., *Mammary Tumors in Mice*, A.A.A.S., Washington, 1945, 123.

plasm (controls) became somewhat more susceptible than the animals of the most susceptible breeds (English, Flemish, Sable, American Blue and Rex) (Fig. 5). The factor has not been obtained (using identical techniques) from other neoplasms of the mouse or rabbit, nor from the rabbit spleen, skeletal muscle, or rabbit testis. The effect of the material has not in our hands resembled anaphylaxis or sensitization of the usual sort. Necroses do not appear at the site of repeated injections and animals injected weekly for almost a year were still highly susceptible and none died because of the injections. Histologically there seems less necrosis in the tumor tissue of XYZ animals and more cells seem alive. The prolonged storage in the frozen state seems to inactivate or mask such inhibitory factors as may be present in the fresh tumor tissue or the surrounding intercellular fluids.¹²

Kidd,¹¹ using our technique, froze Brown-Pearce tumor tissue and kept it frozen for 1-2 months at -22°C until ready for use. He then injected a saline emulsion of the frozen and non-viable tumor tissue, in the manner of our experiments, 7-20 days prior to intramuscular transplantation of the tumor. Among the 16 "blue cross" control rabbits was one animal at 30-35 days with progressively enlarging primary tumor (6%), none had metastases (0%) and none died from the tumor (0%). Among the 53 "blue cross" XYZ animals (called "immunized animals" by Kidd) there were 18 at 30-35 days with progressively enlarging primary tumors (34%), at least 10 had metastases (19%) and 10 died from metastases (19%). Evaluation of his work is difficult since his experiments were terminated at 30-35 instead of at the usual 60 days, control animals were few and autopsies were incomplete in that no enumerations of the incidence, number and volume of the metastases was recorded. Nevertheless his "immunized" (XYZ injected) animals had significantly more enlarging tumors ($X^2=4.7$, $P=0.035$), and among those with tumors a greater mortality from metastases ($X^2=5.6$, $n=1$, $P=0.02$) than their respective controls. The term, "immunized," used by Kidd with respect to the

XYZ injected animals is, we believe, ill considered.

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TABLE I.
Influence of NaCl on Growth of *E. coli* ds.

Hrs of incubation	No NaCl					0.4% NaCl					1.0% NaCl				
	Concentration of streptomycin, $\mu\text{g/ml}$					Concentration of streptomycin, $\mu\text{g/ml}$					Concentration of streptomycin, $\mu\text{g/ml}$				
	0	5	25	100	500	0	5	25	100	500	0	5	25	100	500
	Turbidity.														
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	0	7	0	0	0	0	11	10	4	0	0	0	10	7	1
25	0	13	2	0	0	0	15	14	13	0	0	1	14	19	12
43	0	18	13	0	0	0	18	19	19	5	0	11	20	19	16
91	0	18	16	9	0	0	26	24	24	20	0	14	28	26	24
139	0	15	13	12	0	0	25	22	21	16	0	17	21	19	18

TABLE II.
Influence of Carbon Sources upon the Growth of *E. coli* ds.

Hrs of incubation	No added carbon source				Glucose				Pyruvate				Fumarate			
					Concentration of streptomycin, $\mu\text{g/ml}$				Concentration of streptomycin, $\mu\text{g/ml}$				Concentration of streptomycin, $\mu\text{g/ml}$			
	0	5	15	25	0	5	15	25	0	5	15	25	0	5	15	25
	Turbidity.															
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	0	1	1	0	0	3	0	0	0	0	2	2	0	0	0	2
24	0	10	4	0	0	23	14	5	0	2	19	23	0	1	12	17
41	0	18	18	13	0	36	38	35	0	19	38	42	0	11	26	25
95	0	21	22	18	0	44	45	43	0	27	50	52	0	22	38	40
119	0	20	21	16	0	48	49	46	0	33	52	53	0	23	40	43

TABLE III.
Influence of Carbon Sources in a Synthetic Medium upon the Growth of *E. coli* ss in Presence of Streptomycin.

Incubation, hr	Glucose		Pyruvate		Fumarate	
	Concentration of streptomycin, $\mu\text{g/ml}$		Concentration of streptomycin, $\mu\text{g/ml}$		Concentration of streptomycin, $\mu\text{g/ml}$	
	0	5	0	5	0	5
	Turbidity					
24	0	0	0	0	0	0
48	0	0	10	0	4	0
72	2	0	14	0	9	0
96	21	0	11	0	9	0
120	25	0	14	0	13	0
142	26	0	16	0	15	0
169	30	0	20	0	18	0
193	32	0	21	0	23	0
265	35	0	27	0	28	0
336	36	0	30	0	33	0
528	39	0	43	0	42	0

5 $\mu\text{g/ml}$ not only had no delaying effect but proved to be more favorable to growth. These results thus tend to prove that pyruvate and fumarate favor the growth of both *E. coli* ss and *E. coli* ds, in the presence of streptomycin.

Use of synthetic media for studying the effect of carbon sources upon streptomycin activity on various E. coli strains. In order to eliminate the interfering action of peptone and meat extract upon the effect of supple-

mentary addition of carbon sources, a synthetic (Koser's) medium was used. This medium consisted of 1 g $(\text{NH}_4)_2\text{H}_2\text{PO}_4$, 1 g K_2HPO_4 , 0.2 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g NaCl, 1000 ml distilled water, and was adjusted to pH 7.3. The carbon sources, previously sterilized by passage through a Seitz filter, were added to make a final concentration of 0.2%. Several strains of *E. coli* of varying sensitivity to streptomycin were used in these

Effect of Nutrients Upon Growth of Streptomycin-Sensitive, -Resistant- and -Dependent Strains of *Escherichia coli*.^{*†}

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Among the various constituents of the medium which influence the antibacterial potency of streptomycin, salt, glucose and certain organic acids were found^{1,2} to be of special importance. The presence in nutrient broth of pyruvic and fumaric acids in 1% concentrations enabled *Escherichia coli* to grow in the presence of 10 μ g/ml of streptomycin, whereas in the same medium free from these acids, the organism was inhibited even by 1 μ g/ml of the antibiotic. With the isolation of streptomycin-dependent strains of this organism, it was considered of importance to determine how the growth of such dependent strains (*E. coli* ds) would be affected by the presence in the medium of those substances which normally favor growth of the original streptomycin-sensitive cultures (*E. coli* ss) in the presence of streptomycin.

Effect of NaCl upon the growth of E. coli ds. Varying amounts of NaCl, sterilized by autoclaving, and streptomycin pasteurized by heating for 30 minutes at 60°C, were added to sterile nutrient broth (0.5% peptone and 0.3% meat extract in distilled water) in tubes. These were inoculated with a suspension of *E. coli* ds grown on nutrient agar containing 10 μ g/ml streptomycin. Each tube was thus inoculated with 44,000 viable cells. The cultures were incubated at 28°C and the amount of growth was measured by using a Cenco-Sheard-Sanford Photometer

type B2 with a red filter. Growth was expressed as the average turbidity (100-transmittancy) of three tubes.

E. coli ds did not grow in the media free from streptomycin; in the presence of streptomycin, however, good growth occurred (Table I). The presence and concentration of NaCl had an important effect. Without the salt, growth was poorer and was more sensitive to higher concentrations of streptomycin, so that 500 μ g/ml was sufficient to inhibit completely the growth of the organism. With an increase in concentration of NaCl to 1%, there was a tendency for more rapid initial growth with increasing concentrations of streptomycin. In other words, the presence of NaCl exerts a favorable effect upon the growth of both *E. coli* ss and *E. coli* ds in the presence of streptomycin. The injurious effect of streptomycin upon *E. coli* ss is reduced by the presence of NaCl, and growth of *E. coli* ds is favored.

Influence of carbon sources upon the growth of E. coli ds. A study was made next of the effect of glucose and of salts of pyruvic and fumaric acids upon the growth of *E. coli* ds (Table II). The above nutrient broth (free from NaCl) was used. When no other carbon source was added, growth was rather limited; an increase in the concentration of streptomycin above 5 μ g/ml had a somewhat delaying and even depressing effect upon the growth of the organism. When glucose was added to the broth there was a marked increase in the amount of growth produced; increasing the concentration of streptomycin above 5 μ g/ml had again a delaying effect upon the rate of growth. Pyruvate and fumarate also exerted a highly favorable effect upon the growth of *E. coli* ds. This was especially true of the pyruvate; in this case, however, an increase in the concentration of the streptomycin above

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† Partly supported by a grant made by the Commonwealth Fund of New York.

¹ Green, S. R., and Waksman, S. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 281.

² Green, S. R., Iverson, W. P., and Waksman, S. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 285.

TABLE VI.
Growth of *E. coli* rs* in a Synthetic Medium Containing Different Carbon Sources and Various Concentrations of Streptomycin.

Incubation, hr	Glucose			Pyruvate			Fumarate		
	Concentration of streptomycin, $\mu\text{g/ml}$			Concentration of streptomycin, $\mu\text{g/ml}$			Concentration of streptomycin, $\mu\text{g/ml}$		
	0	5	500	0	5	500	0	5	500
				Turbidity					
98	0	0	0	0	0	0	0	0	0
122	0	0	0	0	0	0	0	0	4
146	0	0	0	0	0	0	0	0	8
168	0	0	0	0	0	0	0	0	13
192	0	0	0	0	0	0	0	0	19
213	0	0	0	0	0	4	0	0	19
238	0	0	0	0	0	10	0	0	20
265	0	0	0	0	0	15	0	0	21
290	0	0	0	0	0	15	0	0	20
362	0	0	0	0	0	15	0	0	15
432	0	0	0	0	0	22	0	0	13
624	0	0	0	0	0	16	0	0	13

* This strain was isolated from the plating of *E. coli* ss in agar containing streptomycin (15 $\mu\text{g/ml}$).

using nutrient agar to which 15 μg /streptomycin had been added. Of the colonies which appeared on the plate, one was found to be resistant to streptomycin. Of the remaining ten, 5 were similar to the first in resistance to streptomycin (in other words, they were *E. coli* rs strains) and 5 were dependent on streptomycin (*E. coli* ds strains). The first colony gave rise to a culture which grew only in synthetic media in the presence of pyruvate or fumarate as a carbon source and with streptomycin only in concentrations of 500 $\mu\text{g/ml}$ (Table VI); no growth took place in higher (1000 $\mu\text{g/ml}$) or lower (100 $\mu\text{g/ml}$) concentrations. When such growth in the pyruvate or fumarate tubes was streaked on plain nutrient agar or on agar containing 10 $\mu\text{g/ml}$ of streptomycin, all grew on the streptomycin agar but not on the streptomycin-free agar. Two of the 3 pyruvate tubes also gave some growth on the plain agar. When this freshly isolated *E. coli* ds was inoculated into nutrient broth, allowed to grow for 4 days at 28°C, then plated out on plain agar and on streptomycin-containing (10 $\mu\text{g/ml}$) agar, 1 ml of the culture contained 137,000,000 sensitive cells and 470,000 dependent cells of *E. coli*. A streptomycin-resistant strain was thus transformed, by growing in a fumarate or pyruvate medium, first into a streptomycin-dependent and then into a streptomycin-sensitive strain.

A preliminary experiment in which various amino acids were added to the synthetic medium with glucose as a carbon source seemed to indicate that l-lysine was necessary for the growth of this resistant organism.

These results thus bring out emphatically the relationship between the nutrition of *E. coli* and its sensitivity or resistance to or dependence on streptomycin.

Summary. 1. Growth of a streptomycin-dependent strain of *E. coli* in nutrient broth was favored by the presence of sodium chloride.

2. Glucose, pyruvate, and fumarate, when added to nutrient broth minus sodium chloride, caused greater growth of the streptomycin-dependent strain. With nutrient broth alone and nutrient broth plus glucose, the greatest initial growth took place in the presence of 5 μg streptomycin/ml. Nutrient broth plus the sodium salts of pyruvate and fumarate, however, favored a greater initial growth in higher concentrations of streptomycin (25 $\mu\text{g/ml}$).

3. In a synthetic medium containing glucose, pyruvate and fumarate as carbon sources, the growth of the sensitive parent *E. coli* strain was inhibited by 5 $\mu\text{g/ml}$; the dependent strain grew in all concentrations of streptomycin from 5 to 1000 $\mu\text{g/ml}$, but not in media without streptomycin. One resistant strain grew in all concentrations of

TABLE IV.
Influence of Carbon Sources in a Synthetic Medium upon the Growth of *E. coli* ds in Presence of Streptomycin.

Incubation, hr	Glucose				Pyruvate				Fumarate			
	Concentration of streptomycin, $\mu\text{g/ml}$				Concentration of streptomycin, $\mu\text{g/ml}$				Concentration of streptomycin, $\mu\text{g/ml}$			
	5	50	500	1000	5	50	500	1000	5	50	500	1000
	Turbidity											
24	0	0	0	0	0	0	0	0	0	0	0	0
49	0	0	0	0	1	1*	0	0	0	0	0	0
66	0	0	0	0	4	5	0	0	0	2†	4	1†
90	0	0	0	0	13	13	0	0	1*	5	9	5
118	0	0	0	0	16	15	10	0	3	10	15	14
138	0	0	1*	0	18	16	8	0	5	13	19	18
164	0	13	10	0	19	18	17	4†	7	16	22	22
193	2*	23	20	0	22	20	19	16†	10	20	23	23
261	23*	28	22	21*	24	22	21	10*	14	27	23	21
384	24	32	26	23	26	25	24	21	22	26	16	15
648	30	36	30	30	38	37	18	15	32	30	16	15

Measurements reported are averages of 3 tubes, except those indicated by * where growth occurred in 2 tubes only and † with growth in one tube only. No growth in media free from streptomycin.

TABLE V.
Influence of Carbon Sources in a Synthetic Medium upon the Growth of *E. coli* rs† in Presence of Streptomycin.

Incubation, hr	Glucose				Pyruvate						Fumarate				
	Concentration of streptomycin, $\mu\text{g/ml}$														
	0	5	50	500	1000	0	5	50	500	1000	0	5	50	500	1000
	Turbidity														
31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	1*	1	0	0	1†	1*	1*	0	0
71	0	0	0	0	0	11	16	16	4	0	5	7	8	7	2*
96	0	0	0	0	0	19	18	20	16	0	10	11	12	13	8
120	0	0	0	0	0	22	20	23	18	7	15	14	16	17	16
144	13†	0	0	0	0	24	22	26	23	17	18	18	22	22	21
168	16	9*	6†	0	0	28	24	27	25	21	21	21	24	24	23
240	24	25	22	0	26†	33	29	32	29	26	27	27	32	28	24
336	28	31	27	26†	24	36	32	34	30	26	29	25	25	19	15
528	31	35	31	34†	31†	44	40	41	26	16	34	30	26	19	16

† This strain was obtained from a culture of *E. coli* ss growing in presence of streptomycin.

experiments, namely the streptomycin-sensitive (ss), streptomycin-resistant (rs) and streptomycin-dependent (ds).

Growth of *E. coli* ss was inhibited by 5 $\mu\text{g/ml}$ of streptomycin, as shown in Table III. The presence of pyruvate and fumarate in the medium did not overcome the inhibiting effect of the antibiotic in the above concentration. *E. coli* ds grew well in all tubes containing streptomycin, varying in concentration between 5 to 1000 $\mu\text{g/ml}$ (Table IV). With the higher amounts of streptomycin, growth with glucose was somewhat better than with pyruvate and fumarate. As one would expect, no growth occurred in the media free from streptomycin.

A streptomycin-resistant strain of *E. coli*

(rs) was obtained by allowing the parent culture to grow in nutrient broth containing 1 $\mu\text{g/ml}$ of streptomycin, plating out the culture at the end of a month's incubation, using agar media containing 1000 $\mu\text{g/ml}$ streptomycin, and isolating one of the colonies. This culture of *E. coli* rs grew best with pyruvate as a source of carbon, both in the absence and in the presence of streptomycin (Table V). An increase in concentration of the antibiotic resulted in a delaying effect upon growth of the culture in the presence of glucose, and to a lesser extent in the presence of the salts of the organic acids.

A second resistant strain was obtained by plating out a 28 hour old nutrient broth culture of the parent sensitive strain of *E. coli*

TABLE VI.

Growth of *E. coli* rs* in a Synthetic Medium Containing Different Carbon Sources and Various Concentrations of Streptomycin.

Concentrations of Streptomycin									
Incubation, hr	Glucose			Pyruvate			Fumarate		
	Concentration of streptomycin, $\mu\text{g/ml}$			Concentration of streptomycin, $\mu\text{g/ml}$			Concentration of streptomycin, $\mu\text{g/ml}$		
	0	5	500	0	5	500	0	5	500
	Turbidity								
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146	0	0	0	0	0	0	0	0	8
168	0	0	0	0	0	0	0	0	13
192	0	0	0	0	0	0	0	0	19
213	0	0	0	0	0	4	0	0	19
238	0	0	0	0	0	10	0	0	20
265	0	0	0	0	0	15	0	0	21
290	0	0	0	0	0	15	0	0	20
362	0	0	0	0	0	15	0	0	15
432	0	0	0	0	0	22	0	0	13
624	0	0	0	0	0	16	0	0	13

* This strain was isolated from the plating of *E. coli* ss in agar containing streptomycin (15 $\mu\text{g/ml}$).

using nutrient agar to which 15 μg /streptomycin had been added. Of the colonies which appeared on the plate, one was found to be resistant to streptomycin. Of the remaining ten, 5 were similar to the first in resistance to streptomycin (in other words, they were *E. coli* rs strains) and 5 were dependent on streptomycin (*E. coli* ds strains). The first colony gave rise to a culture which grew only in synthetic media in the presence of pyruvate or fumarate as a carbon source and with streptomycin only in concentrations of 500 $\mu\text{g/ml}$ (Table VI); no growth took place in higher (1000 $\mu\text{g/ml}$) or lower (100 $\mu\text{g/ml}$) concentrations. When such growth in the pyruvate or fumarate tubes was streaked on plain nutrient agar or on agar containing 10 $\mu\text{g/ml}$ of streptomycin, all grew on the streptomycin agar but not on the streptomycin-free agar. Two of the 3 pyruvate tubes also gave some growth on the plain agar. When this freshly isolated *E. coli* ds was inoculated into nutrient broth, allowed to grow for 4 days at 28°C, then plated out on plain agar and on streptomycin-containing (10 $\mu\text{g/ml}$) agar, 1 ml of the culture contained 137,000,000 sensitive cells and 470,000 dependent cells of *E. coli*. A streptomycin-resistant strain was thus transformed, by growing in a fumarate or pyruvate medium, first into a streptomycin-dependent and then into a streptomycin-sensitive strain.

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3. In a synthetic medium containing glucose, pyruvate and fumarate as carbon sources, the growth of the sensitive parent *E. coli* strain was inhibited by 5 $\mu\text{g/ml}$; the dependent strain grew in all concentrations of streptomycin from 5 to 1000 $\mu\text{g/ml}$, but not in media without streptomycin. One resistant strain grew in all concentrations of

streptomycin as well as in media without streptomycin, while a second resistant strain grew only in a streptomycin concentration of 500 $\mu\text{g/ml}$ with pyruvate and fumarate as carbon sources.

4. This second streptomycin resistant strain was transformed into a streptomycin-de-

pendent strain under the above conditions. The dependent strain so obtained remained dependent after several transfers on nutrient agar containing streptomycin, and in turn gave rise to streptomycin-sensitive cells when a large inoculum was placed in streptomycin-free nutrient broth.

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A Simple Technic for Counting Megakaryocytes.*

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Principle of method. Make a thick drop preparation of an accurately measured volume of marrow as in examination for malaria and count all the megakaryocytes in the preparation.

Method. From a well-shaken, evenly suspended marrow specimen,¹ fill a Sahli hemoglobin pipette to the 20 cmm mark and blow contents onto a clean slide to form a thick drop. Spread evenly with the pipette tip over an oval area about 1.5×3 cm. Usually several such thick drop slides are made so that additional slides are available for examination in case part of the drop is detached by too vigorous washing and so that, when the count is low, a statistically significant number of cells can be counted.¹ Place slides in an incubator overnight. The following morning, immerse the slides in a solution of 5% formalin in 1% acetic acid in a Coplin jar until the erythrocytes are lysed and the thick drop is grayish in color. Flood the laking solution off the slide with water and gently rinse with buffer-phosphate solution (pH 6.4).² Stain with Wright's stain alone for 2 to 3 minutes. Add buffer-phosphate solution and stain 60 to 90 minutes. Wash slide by holding hori-

zontally under gently running water. Air dry. Spread a thin film of immersion oil over the smear and examine systematically, using a mechanical stage and $200\times$ magnification, preferably with an 8 mm objective which is not immersed in the oil. Count all megakaryocytes seen in the specimen and multiply by 50 to express as number per ml. If the total nucleated marrow cell count per cmm and the leukocytic-erythrocytic ratio are determined, the number of megakaryocytes per million nucleated marrow cells or per million nucleated erythrocytic cells may be calculated. Morphology is fairly well preserved and any cell can be examined in greater detail by switching to the oil immersion lens.

In previously reported methods a count was made of all megakaryocytes and all nucleated marrow cells³ or of all megakaryocytes and all nucleated erythrocytic cells⁴ in an area of arbitrarily determined size on a thin marrow smear and results expressed, respectively, as megakaryocytes per million nucleated marrow cells or per million nucleated erythrocytic cells. These methods have the disadvantages that they are extremely time-consuming and that such large cells are seldom distributed evenly and, indeed, are likely to be found in greatest numbers at the tails of the smear. The area selected for examination will thus

* Aided by a grant from the George A. Myers Research Fund for Study of Hemorrhagic Diseases.

¹ Osgood, E. E., and Seaman, A. J., *Physiol. Rev.*, 1944, **24**, 46.

² Osgood, E. E., "Laboratory Diagnosis," p. 478, 3rd ed., Blakiston.

³ Limarzi, L. R., and Schleicher, E. M., *J. A. M. A.*, 1940, **114**, 12.

⁴ Dameshek, W., and Miller, E. B., *Blood*, 1946, **1**, 27.

greatly influence the count. If results are expressed as number per million nucleated erythrocytic cells, megakaryocytes are actually more numerous than results would indicate in diseases characterized by erythrocytic hyperplasia such as blood loss anemia due to purpura hemorrhagica and actually less numerous than the results would indicate in erythrocytic hypoplasia. The same objections apply when the result is expressed as number per million nucleated marrow cells and, in addition, leukocytosis, leukemia, and other hyper- or hypoplastic diseases of the

leukocytic system would distort the apparent number of megakaryocytes. For these reasons, it would be preferable to determine the absolute number per ml.

Study by this thick drop technic of the number of megakaryocytes found in normal and pathologic marrows is under way, but not yet complete. Preliminary results would seem to indicate that the normal range is between 500 and 4000 megakaryocytes per ml; between 20 and 150 per million nucleated marrow cells; and between 50 and 300 per million nucleated erythrocytic cells.

16797

Influence of Amino-Acids on Adrenal Enlargement, Nephrosclerosis and Hypertension by Anterior Pituitary Preparations.*

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Previous investigations carried out in this laboratory have shown that overdosage with lyophilized anterior pituitary (LAP) can produce nephrosclerosis and hypertension in rats fed a 30% casein diet.¹ It was also observed that different kinds of proteins² and protein hydrolysates³ are as effective as casein in the production of nephrosclerosis and hypertension by LAP. This paper is concerned with the nephrosclerotic activity of LAP in animals fed a diet in which the protein component was provided by a mixture of crystalline amino-acids.

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¹ Dontigny, P., Hay, E. C., Prado, J. L., and Selye, H., *Am. J. Med. Sciences*, 1948, **215**, 442.

² Hay, E. C., Prado, J. L., and Selye, H., *Canad. J. Research (Section E, Med. Sc.)*, 1948, **20**, 212.

³ DeGrandpré, R., Prado, J. L., Dontigny, P., Ledue, J., and Selye, H., *Fed. Proc.*, 1948, **7**, 27.

Material and methods. 25 hooded black-and-white male rats weighing 110 to 130 g were divided into 4 groups. Groups I and II received a "22%" amino-acid[†] diet and Groups III and IV a "22%" Amigen[†] (enzymatic casein hydrolysate) diet, both rations containing 4% sodium chloride. In order to maintain equal food intake, all animals received their diet as a 70 g % aqueous suspension, 3 times daily at 6-hour intervals through a stomach tube. First Groups I and II were adapted to this procedure by forced-feeding mixtures of Amigen and amino-acids in which the proportion of Amigen was gradually decreased so that on the 10th day only amino-acids were fed. At the same time, Groups III and IV were adapted to forced feeding with the Amigen diet. The volume and caloric value of the food given was the same in all groups. The amount of food was so adjusted that the animals slightly increased in weight.

After the adaptation period, all animals

[†] "22%" means a nitrogen content corresponding to 22% casein. For composition of diets see Tables I and II.

TABLE I.
Composition of Amino-acids Mixture.

	g
L-(+)-arginine hydrochloride	5.8
L-(+)-histidine	11.9
L-(+)-lysine	30.0
Dl-isoleucine	24.0
L-(—)leucine	21.6
Dl-methionine	14.4
Dl-phenylalanine	16.3
Dl-threonine	23.8
Dl-tryptophane	4.8
Dl-valine	33.6
L-(+)-glutamic acid	137
L-(—)-tyrosine	20.0

TABLE II.
Composition of Diets (Parts %).

	Amigen	Amino-acids diet
Amigen	30*	—
Amino-acids mixture	—	37*
Corn starch	28	28
Sugar	30	30
Cod liver oil	1	1
Fat	1	1
Steenbock's mineral mixture	4	4
Sodium chloride	4	2.5
Cellu flour	1	1
Charcoal	5	5
Vitamins†	10 ml	10 ml
Sodium bicarbonate†	—	21.3

* These amounts correspond in nitrogen to 22 g of casein.

† Used for neutralization of the amino-acids hydrochlorides.

were castrated and unilaterally nephrectomized. LAP treatment was started the following day in Groups I and III, and continued for 27 days. LAP was administered in 2 daily subcutaneous injections of 0.1 cc of 20 mg/day during 15 days, this dose being raised to 30 mg for 6 days and to 40 mg during the last 6 days.

The blood pressures were determined on the 27th day of treatment by the direct method, as previously described,¹ the animals being killed next day.

The severity of nephrosclerosis was designated in terms of a scale of 0 to ++++. The criteria employed microscopically were arteriolonecrosis, capillary tuft hyalinization and cast formation with resultant tubule dilatation and atrophy.

Discussion. As may be seen from Table III, adrenal enlargement, nephrosclerosis and hypertension can be produced by LAP, on a

TABLE III.
Effect of Amino-acids Content of the Diet on Hypertension Produced by LAP.*

Groups	No. rats	Treatment	Body wt		Nephrosclerosis†		Adrenal wt, mg	Kidney wt, mg/100 cm ² b. surface	Blood pressure, mm Hg.
			Initial	Final	Incidence, %	Severity, %			
1	6	“22%” amino-acids + LAP	128	189 ± 3.5	50	22	72 ± 6.1	663 ± 51	143.8 ± 20
2	6	“22%” amino-acids	126	169 ± 2.2	0	0	32 ± 1.2	420 ± 18	115.5 ± 13.9
3	6	“22%” Amigen + LAP	126	225 ± 3.0	100	75	92 ± 11	629 ± 18	140.6 ± 6.3
4	7	“22%” Amigen	124	194 ± 2.2	0	0	39 ± 1.3	324 ± 11.3	129.4 ± 10.8

* Lyophilized anterior pituitary.

† “22%” means a nitrogen content corresponding to 22% casein.

‡ Nephrosclerosis as observed by microscopic examination.

diet in which amino-acids completely replace proteins. This finding indicates that the constituent amino-acids are responsible for the conditioning, by dietary proteins, to the above toxic effects of LAP overdosage. Our data also show that the growth, adrenal enlargement and nephrosclerosis produced by LAP were less pronounced on the amino-acid diet than on the Amigen diet of equal nitrogen content. It may incidentally be mentioned that the usual myocarditic changes³ which accompany this type of hypertension were also less obvious in the amino-acid than in the Amigen groups. This could possibly be explained by the fact that the animals of Groups I and II received an unnatural mixture of amino-acids. Yet

³Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical Physiological Chemistry*, The Blakiston Co., Philadelphia, 1947.

³Selye, H., *J. Clin. Endocrinol.*, 1946, 6, 117.

the existing difference between Groups I and III is of sufficient magnitude to suggest that equal amounts of amino-N are not necessarily equally conducive to the production of nephrosclerosis by LAP.

Summary. Adrenal enlargement, nephrosclerosis and hypertension were produced by a lyophilized anterior pituitary (LAP) preparation in rats kept on a synthetic diet containing no protein, but an adequate amount of amino-acids. The severity of the lesions was less marked, however, than in rats receiving an equivalent amount of nitrogen in the form of a casein-hydrolysate. It appears that amino-acids suffice to sensitize the organism to the production of hypertensive disease by LAP. This is noteworthy since no such pathologic changes can be produced by LAP on other diets deficient in proteins.

16798

Comparative Assays of Rodenticides on Wild Norway Rats.

II. Acceptance.*

SALLY H. DIEKE. (Introduced by C. P. Richter.)

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In the previous paper of this series,¹ 8 common rodenticides were compared on the basis of their acute toxicities to recently trapped wild Norway rats. As was pointed out in that paper, a high toxicity does not, however, guarantee effectiveness under field conditions; of at least equal importance are those properties, such as a bitter taste or the lack of it, which influence the voluntary consumption of poisoned baits. It is the purpose of the present work to reexamine the same 8 rodenticides in the light of their acceptability and

effectiveness when offered to wild Norway rats in a standard bait under controlled conditions in the laboratory.

Methods. The 565 wild Norway rats on which data are here given were trapped in the city of Baltimore during the period from July 1946 to December 1947. Before use they were kept in the laboratory, in large holding cages, for a few days; this delay proved necessary to allow for occasional deaths or for signs of debility to become evident. They were maintained on Purina fox chow and water during this time. The healthy specimens were then transferred (in almost every instance within 2 weeks after trapping) to individual wire mesh cages provided with a metal food cup and a water bottle. The food cup had fitted over it an aluminum

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

¹Dieke, S. H., and Richter, C. P., *Pub. Health Rep.*, 1946, 61, 672.

cover with a small oval opening, designed to minimize spillage, and it rested directly under a matching hole cut in one corner of the wire mesh bottom of the living compartment.

The bait used was a special yellow corn (Funks' Hybrid G-94, grown in McLean County, Illinois†), freshly ground to a uniform fineness for each week's work. The cornmeal was weighed into the food cups and placed in the cages at 4:30 P.M. for the rats to eat overnight. Next morning at 9:30 a.m. the food cups were removed and reweighed. Extra cups containing cornmeal were occasionally weighed in the afternoon and again next morning to make sure that atmospheric conditions did not cause changes in weight which might reduce the accuracy of the observations. No changes of more than 0.2 grams ever occurred, which was considered within the experimental error.

The cups were marked and always returned to the same cage. The rats were purposely left without food during the day in order to prevent them from starting to eat before dusk, which is their normal feeding time under field conditions.

After 2 nights of prebaiting with plain corn, according to the above schedule, poison was added to the cornmeal for the third night. To ensure a uniformly small particle size, each sample was first ground in a mortar for 2 or 3 minutes; it was then added to the appropriate amount of cornmeal and blended in a mechanical mixer for 15 to 20 minutes. The poisoned baits were always freshly prepared on the day the rats received them.

Next morning the poisoned food was removed and weighed, and any rats alive at this time were fed fox chow until they died or were discarded (4 to 10 days after poisoning, depending on the poison and the condition of the rat). A large number of rats were autopsied, including all those not dying promptly, in order to exclude data on rats not succumbing to the poison alone. No surviving rat was ever used again.

Most of the experiments were performed during the autumn, winter and spring months.

No seasonal variation in toxicity was found in previous work, and since the present work likewise gave no indication of any, the data have been considered as a whole. A difference in response attributable to sex was previously found to exist for none of the poisons except red squill; so a separation of data on male and female rats is here made only for that poison. And although data on young rats are included in the table giving nightly consumptions of plain corn, no sexually immature rats were used in the poison experiments, thus obviating any variation attributable to age.

The poisons used came from the same containers as in the preceding work, with the exception of sodium fluoroacetate (1080) and arsenic trioxide, supplies of which had been exhausted. The new samples of these two poisons were reassayed by stomach tube, according to the method previously described,¹ and 52 additional wild Norway rats were used to check the toxicity figures previously obtained for the other poisons. No substantial changes in toxicity were detected and accordingly the LD50 figures previously obtained for strychnine sulfate, alpha-naphthyl thiourea (ANTU), thallium sulfate, fortified red squill and barium carbonate were allowed to stand.

Results. Plain Cornmeal. The average amounts of unpoisoned cornmeal eaten by the rats during the prebait period are shown in Table I. They have been broken down according to size of rat and sex, to show that the average consumption increased from 6 to 10 g for immature rats up to more than 18 g for full grown males. Individual large rats had intakes as high as 30 g on the second night, and a few ate more than 25 g both nights.

In each weight range the females seem to have eaten somewhat less than the males, although not significantly less in the young adult classes (100 to 299 g body weight). On the average all but the small rats ate more the second night than they did the first, indicating a certain hesitation to eat what was presumably an unfamiliar food. Only 24 rats (not included in the tabulation) refused

† Provided through the courtesy of Funk Brothers Seed Company, Bloomington, Ill.

TABLE I.
Consumption of Cornmeal on 2 Successive Nights by 565 Wild Norway Rats.*

Wt range	No. rats	Avg body wt, g	Avg consumption, g	
			1st night	2nd night
Below 50 g	4 ♂	47.0	8.0	7.1
	3 ♀	47.0	6.3	6.0
50-99	49 ♂	80.0	10.3	10.0
	63 ♀	73.8	9.7	9.4
100-199	51 ♂	140.5	10.7	11.1
	86 ♀	144.5	10.3	11.2
200-299	74 ♂	249.0	12.7	14.6
	77 ♀	251.5	13.5	14.5
300-399	63 ♂	342.1	15.6	17.8
	60 ♀	343.4	13.9	16.4
400-499	19 ♂	435.5	16.4	18.4
	13 ♀	427.4	14.3	16.4
>500	3 ♂	543.0	11.8	17.0
	0 ♀	—	—	—

* Not including data on 23 additional rats which refused to eat the first night, and 1 rat which refused both nights.

to eat any cornmeal on the first night, a ratio of approximately one refusing rat in 24, or 4%, and of these all but one ate well the second night.

Poisons. The kills obtained with various percentages of each poison are given in Table II, which also lists for comparison the acute median lethal doses (LD50's) of the poisons as determined by stomach tube administration. The lowest poison concentrations at which complete kills were obtained are seen to range from 0.1% for thallium sulfate to 5% for female rats receiving squill. No bait containing strychnine sulfate or barium carbonate killed all the rats receiving it, nor was any concentration of red squill completely successful against both male and female rats.

In general, the higher the concentration of any poison, the more rats died from eating it. This was, however, not true for strychnine sulfate and squill: for these, some fatalities occurred throughout the range but increasing the concentration did not increase the efficiency of the bait.

The reduced consumptions of bait resulting from the addition of the various poisons to the cornmeal are shown in Table III, which gives data on the same rats as Table II.

This shows that the amount of bait consumed decreased fairly regularly with increasing content of poison. At the levels marked with asterisks, which were the lowest concentrations killing all the rats used, the average intakes were reduced to 14% of pre-bait for 1080, 21% for female rats receiving squill, 28% for ANTU, and 30% for zinc phosphide. No reduction at all followed the introduction of thallium sulfate into the bait at the equivalently lethal concentration. Reduced intakes are seen also for strychnine and barium carbonate, but even with these poisons a total refusal to eat never occurred.

Discussion. From a practical standpoint, experiments such as these serve only to indicate what will happen under field conditions. Just as toxicity figures based on administration by stomach tube represent the irreducible minimum of poison which is likely to kill, so results with trapped and individually caged wild Norways merely show what percentages of poison in bait can be expected to kill under the most favorable conditions in the field. Captive wild rats have been removed from their normal environment and may well be suspicious of any food offered them; on the other hand they are not subject to distract-

RODENTICIDES TESTED ON WILD RATS

TABLE II.
Comparative Killing Concentrations.

(Voluntary consumption of poisons in cornmeal bait by wild Norway rats; mortality ratios (rats dying/rats used) for each poison are listed opposite the appropriate concentrations. The median lethal doses and their standard errors are added for comparison at the bottom of the table.)

Conc. in bait	1080 (36 rats)	Strychnine SO ₄ (20 rats)	ANTU (36 rats)	Tl ₂ SO ₄ (36 rats)	Zn ₃ P ₂ (40 rats)	As ₂ O ₃ (24 rats)	Squill (24 ♀, 24 ♂)	BaCO ₃ (20 rats)
.01%	2/ 4							
.02	1/ 4		0/4	0/ 4				
.05	10/12	1/4	4/8	11/12	2/8			
.1	7/ 8	0/4	1/4	8/ 8	1/4			
.2	8/ 8	5/8	5/8		8/8			
.5		0/4	4/4	8/ 8		0/4		
1			8/8	4/ 4	4/4	1/4		
2					7/8	6/8	5/8 1/8	
4						4/4		
5					8/8		4/4 1/4	
6						4/4		
10							2/4 0/4	0/4
15								3/4
20							2/4 0/4	3/4
50							2/4 2/4	7/8
Lowest conc. killing all rats, %	0.2	—	0.5	0.1	0.2	4	5	—
LD ₅₀ Acute,* mg/kg (± S.E.)	0.44 ±0.19	4.8 ±0.4	6.9 ±0.5	15.8 ±0.9	40.5 ±2.9	57.5 ±7.0	133 ±10 276 ±29	1480 ±340

* Values for 1080 and As₂O₃ from Table IV, the rest from reference 1.

TABLE III.
Average Voluntary Intakes of Poisoned Cornmeal Showing Lowered Consumptions with Increasing Concentrations of Poisons.*

Conc. in bait, %	1080 g	Strychnine SO ₄ g	ANTU g	Tl ₂ SO ₄ g	Zn ₃ P ₂ g	As ₂ O ₃ g	Squill ♀, g ♂, g	BaCO ₃ g
0 (prebait)	15.4	16.0	14.5	15.2	13.8	15.6	16.4 17.4	14.7
.01	9.1							
.02	2.6		9.0	11.0				
.05	3.3	7.7	8.0	15.7	10.3			
.1	2.4	6.1	5.7	15.5*	6.4			
.2	2.2*	4.1	2.7		4.1*			
.5		2.8	4.1*	8.4		2.9		
1			1.2	6.9	2.5	0.9		
2					1.7	1.7	9.7 8.7	
4						1.4*		
5					1.3		3.4* 4.9	
6						0.6		
10							0.4 3.5	1.5
15								4.0
20							1.0 0.2	2.4
50							0.8 1.3	3.4

* The prebait figure in each case is the average intake of all rats used for that poison. The intakes of poisoned bait at the lowest concentrations giving complete kills are marked with asterisks. (Mortality data on these same rats are given in Table II.)

tions such as other food supplies, or disturbances resulting from attacks by other rats or natural enemies, which could influence their food consumption. It is, for instance, unlikely that ever unwanted rat would have

the opportunity or even the inclination to eat 15 or 20 g of a 0.1% thallium sulfate bait at one sitting, and therefore poisoning with so low a concentration, while very efficient in the laboratory, might easily prove futile for

TABLE IV.

Reassays of 1080 and Arsenic Trioxide.

(Administered by stomach tube, in water containing 10% acacia, to recently trapped wild Norway rats (methods described in Ref. 1).)

Poison and source	Dose, mg/kg BW	Mortality	LD ₅₀ ± S.E. mg/kg BW
1080 (Monsanto Chemical Co.)	0.2	1/4	0.44 ± 0.19
	0.3	2/4	
	0.5	4/7	
	1	4/6	
	2	7/8	
As ₂ O ₃ (Mallinkrodt, analytical)	25	0/4	57.5 ± 7.0
	50	3/8	
	75	3/4	
	100	1/1	

practical purposes. But it can be said that any poison not effective in laboratory feeding tests such as these will undoubtedly kill far fewer rats in the field.

On the basis of the above results, then, 1080, ANTU, thallium sulfate, and zinc phosphide should be good and efficient rodenticides; arsenic trioxide (of good grade and with small particle size) slightly less so; while perfect control of Norway rat populations should not be obtainable through the use of red squill, barium carbonate, or strychnine sulfate.

The value of prebaiting is indicated by the fact that rats did not entirely refuse to eat baits containing the very bitter strychnine sulfate, or even concentrations of red squill as high as 50%, after they had had access to the same (unpoisoned) bait for 2 nights previously.

The reduced intakes of bait after adding poison may be ascribed either to early detection of the poison by the rats and subsequent refusal, or to the rapid onset of toxic reactions making the rats too ill to do more than sample the bait. In the case of 1080, which rats do not taste,² the second is probably the determining factor. For strychnine sulfate, the bitter taste is undoubtedly the deterrent. For most of the others it is more difficult to distinguish between the two possibilities. Thal-

lium sulfate appears to be entirely undetected by the rats, and, were it not for the hazard to humans involved in its use, would seem to be the ideal rat poison.

Summary. The relative efficiencies of 8 common rodenticides were determined by offering the poisons to recently trapped wild Norway rats in a standard bait (freshly ground cornmeal) under standard conditions. The voluntary consumption of baits containing sodium fluoroacetate (1080), alpha-naphthyl thiourea (ANTU), thallium sulfate and zinc phosphide resulted in complete kills at concentrations of 0.5% or lower. A sample of arsenic trioxide of relatively high toxicity required a concentration of 4% for a complete kill. All the female rats offered 5% of a good grade of fortified red squill died, but higher concentrations, and all concentrations offered to male rats, gave only partial kills. Barium carbonate gave good but not complete kills at concentrations up to 50%, while strychnine sulfate killed few rats in the range tested.

The amounts of unpoisoned cornmeal consumed on 2 successive nights, by male and female wild Norway rats in various weight groups were determined. The addition of every poison except thallium sulfate to the cornmeal caused a considerable reduction in intake, owing either to recognition of the poison or to a rapid onset of toxic effects.

² Richter, C. P., in press.

Decreases in Plasma Volume During Electrically Induced Convulsions in Man.

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Electrically induced convulsions as used in the treatment of mental disease consist of a brief period of generalized muscular contractions of maximal vigor; they are associated with marked rises in intrathoracic and intra-abdominal pressures and the development of anoxia. Studies made here on the effects of electroshock therapy have afforded data bearing on problems connected with the physiology of violent physical exercise; it was considered of interest to describe the changes in plasma volume which occur during this type of muscular exertion.

Material and methods. Thirteen subjects were studied, 8 of whom were women. Cardiovascular function was normal except for mild hypertension in several. Two of the subjects were studied twice each, the rest being observed in one treatment each. Three of the women (Cases 11, 12, 13) were studied during convulsions moderated as a consequence of the previous intravenous injection of curare (solution Intocostrin or solution d-tubocurarine Squibb); in one of these (Case 11) observations had also been made during a seizure induced without the previous injection of curare.

The plasma volume was measured by the short indirect method of Gibson and Evans,¹ 4 samples of venous blood being taken over a period of 30 to 40 minutes immediately before the induction of the seizure, and another being taken immediately at the end of the convulsion; in 5 instances an additional sample was taken 15 minutes later. The concentration of dye in each sample of plasma was estimated by means of a Coleman Junior Spectrophotometer. The plasma protein concentration was estimated in 5 patients by means of

the copper-proteinate method;² measurements of plasma protein so made were used to estimate changes in plasma volume using the following formula:

$$\frac{\text{plasma volume after seizure}}{\text{plasma protein after}} = \frac{\text{plasma volume before} \times \text{plasma protein before}}{\text{plasma protein after}}$$

Observations. The plasma volume was diminished at the end of the convulsive seizure in every instance; the decreases ranged between 50 cc and 1040 cc, or 1.7% to 34.7% of the original values (Table I, Fig. 1). In the case of the men, the decreases were between 9.0% and 34.7% of the control levels, the average being 19.4%. The largest decrease occurred in a young muscular athlete in good condition (Case 1), while the smallest decrease in this group was observed in a weak, malnourished, middle-aged individual who had led a sedentary life for several decades (Case 5). One patient, studied twice, showed diminutions in plasma volume of 25.8 and 17.9% on the two occasions. In the uncured women, diminution in plasma volume immediately after the convulsion amounted to between 7.3 and 13.7% of the control values; the average was 10.5%. The curarized women showed decreases of 1.7 to 4.7% of the control values for plasma volume, changes which are not significant; the average change was 3.2%. One woman studied twice showed a decrease in plasma volume of 7.3% when not given curare, and of 3.1% when curarized.

Fifteen minutes after the seizure the plasma volume as a rule was partially restored to its original value (Table I).

Discussion. Kaltreider and Meneely³ re-

¹ Gibson, J. G., II, and Evans, W. A., Jr., *J. Clin. Invest.*, 1937, 16, 301.

² Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M., *Bull. U. S. Army Med. Dept.*, 1943, 74, 66.

TABLE I.
Changes in Plasma Volume and Plasma Protein Concentration After Convulsions.

Case	Sex	Plasma volume				Plasma protein, g %		
		Before, cc	After, cc	Difference, %	15 min. later, cc	Before	After	15 min. later
1	M	3000	1960	34.7	2740	6.86	8.17	7.53
2a	M	3000	2225	25.8				
2b	M	2600	2135	17.9				
3	M	3130	2610	16.6	2900	6.69	7.29	7.08
4	M	3910	3430	12.3	3430	5.45	6.44	6.08
5	M	3050	2775	9.0				
	Avg—Men			19.4				
6	F	1420	1225	13.7				
7	F	1750	1520	13.1				
8	F	2460	2190	11.0				
9	F	2140	1940	9.4	2210	6.27	7.16	6.49
10	F	2360	2160	8.5				
11a	F	2120	1965	7.3				
	Avg—Women			10.5				
	Avg—Men and women			14.9				
11b	F	2130	2065	3.1				
12	F	1910	1820	4.7				
13	F	3010	2960	1.7	2910	5.37	5.64	5.73
	Avg—Curarized women			3.2				

viewed the earlier work on the effect of exercise on the plasma volume and added additional data; much of the previous work was fragmentary or carried out by means of methods of doubtful validity. Indeed, the validity of the method of Gibson and Evans¹ used by Kaltreider and Meneely,³ and also in the present study, has not been established in exercising subjects. In view of the fact that the dye used in that method is attached to albumin,⁴ it is clear that if this protein left the vascular bed during exercise the decreases in plasma volume as measured would be smaller than the actual decreases. It appears, therefore, that the decreases in plasma volume which occur during exertion actually are as large as those reported by Kaltreider and Meneely³ and in the present study, or possibly greater. A criticism of the validity of the method during exertion advanced by Ebert and Stead⁵ is more serious; those authors reported a change in optical quality of the serum after exercise, which in 5 of their 6 experiments was large enough to introduce serious errors into the measurement of plasma

volume with the dye method. These errors were detected by Ebert and Stead⁵ by means of the large discrepancies found when de-

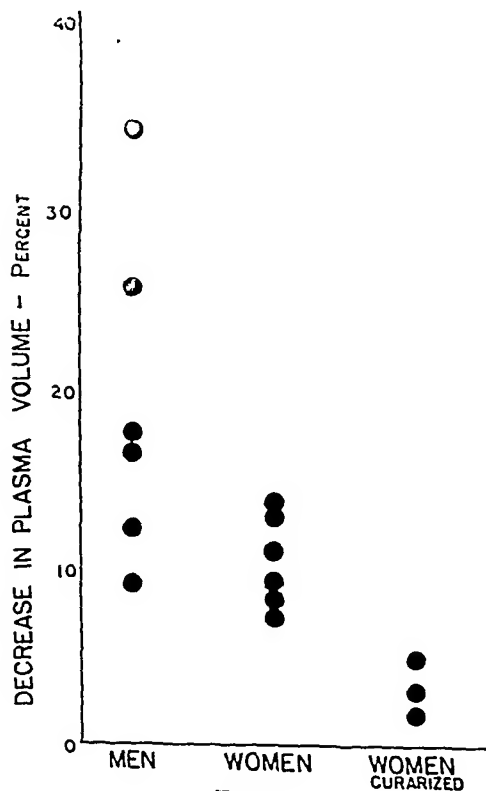


Fig. 1.

³ Kaltreider, N. L., and Meneely, G. R., *J. Clin. Invest.*, 1940, 19, 627.

⁴ Gregerson, M. I., and Gibson, J. G., *Am. J. Physiol.*, 1937, 120, 494.

⁵ Ebert, R. E., and Stead, E. A., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1941, 46, 139.

TABLE II.
Plasma Volume Immediately After Convulsion.

Case	Measured by dye method, cc	Calculated from changes in plasma protein, cc	Avg. deviation, %
1	1960	2480	± 11.7
3	2610	2870	± 4.8
4	3430	3310	± 1.8
9	1940	1870	± 1.8
13	2960	2870	± 1.5

TABLE III.
Plasma Volume 15 Minutes After Convulsion.

Case	Measured by dye method, cc	Calculated from changes in plasma protein, cc	Avg. deviation, %
1	2740	2740	± 0
3	2900	3060	± 2.7
4	3430	3510	± 1.2
9	2210	2050	± 3.8
13	2910	2820	± 1.6

creases in plasma volume during exercise were, on the one hand, measured by the dye method, and on the other hand, calculated from changes in plasma protein concentration. In the present study, a similar comparison was made in 10 experiments. In 4 of the 5 based on studies made immediately after the convulsion there was satisfactory agreement between the two methods, *i.e.*, the plasma volumes calculated by the two methods agreed with each other within 4.8% of the mean of each pair of values; there was no consistent directional difference between the two (Table II). In one of these 5 experiments agreement was not as good, the two values differing from each other by 11.7% of their mean. In a comparison of data obtained 15 minutes after the convulsion in 5 experiments, good agreement was found between plasma volumes as measured by the dye method and those calculated from changes in plasma protein level (Table III). It is concluded, therefore, that the criticism of the dye method in exercise made by Ebert and Stead² is not substantiated by the present data in that whatever changes in optical properties of serum may occur are not significant in measurements of plasma volume by this method; possibly the longer duration of their exercise or the

fact that our subjects were fasting may explain the differences found.

The finding in the present study of decreases in plasma volume in uncurarized subjects of 7.3 to 34.7%, averaging 14.9%, is smaller than expected on the basis of comparison with the data of Kaltreider and Meneely.³ However, conditions in the experiments of these authors were so different from those of the present study as to invalidate direct comparisons. The rises in venous pressure, and presumably also in capillary pressure, were far greater in our experiments than in those of Kaltreider and Meneely;⁴ the increases which occur in uncurarized subjects during electrically induced convulsions average 53 cm of water and in curarized subjects average 23 cm of water.⁵ However, as Landis^{7,8} pointed out, increased filtering pressure accounts for only a small part of the loss of fluid from the circulation during muscular exercise. Moreover, during electrically induced convulsions the rises in venous and capillary pressures which occur do not become increased effective filtering pressures everywhere, because of the simultaneous, approximately equal, rises in intrathoracic,⁹ intra-abdominal⁹ and cerebrospinal fluid pressures.¹⁰ Accordingly, blood drawn after the end of a seizure, as in the present study, is a mixture of some blood exposed to high filtering pressures and some exposed to normal filtering pressures in the small vessels. Accordingly, little significance can be attached to the fact that the average decrease in plasma volume found here in uncurarized subjects, *i.e.*, 14.9%, is close to that found by Landis *et al.*¹¹ *i.e.*, 12.0%, when those authors artificially raised venous pressures in the arm to a level comparable to that reached in patients

² Altschule, M. D., and Tillotson, K. J., *Arch. Neurol. and Psychiat.*, in press.

⁷ Landis, E. M., *Physiol. Rev.*, 1934, 14, 404.

⁸ Landis, E. M., *Ann. New York Acad. Sci.*, 1946, 46, 713.

⁹ Gordh, T., and Silfverskiöld, B. P., *Act. Med. Scandinav.*, 1943, 113, 153.

¹⁰ Marsan, C. A., and Fuortes, M. G. F., *Rev. di Neurol.*, 1947, 17, 1.

¹¹ Landis, E. M., Jonas, L., Augvine, M., and Erb, W., *J. Clin. Invest.*, 1932, 11, 717.

experiencing induced seizures; the similarity of the findings does not constitute an argument for the importance of increases in filtering pressure in causing the changes in plasma volume observed in the present study.

The effects of other factors which operate during induced seizures are similarly impossible to evaluate. These factors include severe anoxia and hypercarbia,¹² muscular hyperemia with consequent increases in filtering surface due to vigorous muscular contractions¹³ and increased transudation of fluid, of uncharacterized etiology, from the vessels of exercising limbs.⁸ Likewise, it is impossible to establish significance of the osmotic effects of muscle cell metabolites, considered to result in the drawing of water from the small blood vessels during exercise.^{7,8} Until the factors which regulate loss of water from the circulation during exercise can be defined precisely, it will not be possible to analyze the mechanisms responsible for the loss of circulating fluid which occurs during the course of electrically induced seizures. Nevertheless,

¹² Altshule, M. D., Sulzbach, W. M., and Tillotson, K. J., *Am. J. Psychiat.*, 1947, **103**, 680.

¹³ Landis, E. M., *Am. J. Physiol.*, 1931, **98**, 704.

the data of the present study show a parallelism between the violence of the convulsions induced and the decreases in plasma volume which occurred; the average decrease in men was 19.4%, in uncured women 10.5% and in cured women 3.2%.

It is clear from the present study that the losses of fluid from the circulation, and their possible consequences, are not essential for obtaining a satisfactory clinical result in patients given electroshock therapy.

Summary and conclusions. Measurements of plasma volume were made 15 times in 13 subjects in whom convulsions were induced electrically for the treatment of mental disease. Decreases in plasma volume were appreciable and varied in amount with the severity of the seizures induced. The previous administration of curare moderated the convulsions and minimized decreases in plasma volume. It is concluded that the method of Gibson and Evans¹ for measuring plasma volume is valid under the conditions of the present study. It is also concluded that the transitory decreases in plasma volume which occur during electrically induced convulsions are a side reaction and are not essential for obtaining a satisfactory clinical result.

16800 P

Urinary Penicillin Excretion in Women.

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The effectiveness of antacids in preventing penicillin destruction by gastric acidity has been measured by studies of the urinary recovery of orally administered penicillin.^{1,2} In these investigations, male subjects had to be studied, since reproducible values were not obtainable with female test persons. This report is concerned with the determination of a

possible relationship between renal clearance of penicillin and the menstrual cycle.

Methods. Three healthy, young, adult women volunteered for these studies. For each test 25,000 units of commercial sodium penicillin were dissolved in 200 cc of an aqueous solution or suspension of 2 g of buffer or antacid and were taken orally 2 hours after a light breakfast consisting of fruit juice, coffee, and a slice of toast. No food was taken in the following 2 hours. The antacids were the same as previously used:¹ sodium

¹ Golden, M. J., and Neumeier, F. M., *Science*, 1946, **104**, 102.

² Perlstein, D., Kluener, R. G., and Liebmann, A. J., *Science*, 1945, **102**, 66.

URINARY EXCRETION IN WOMEN

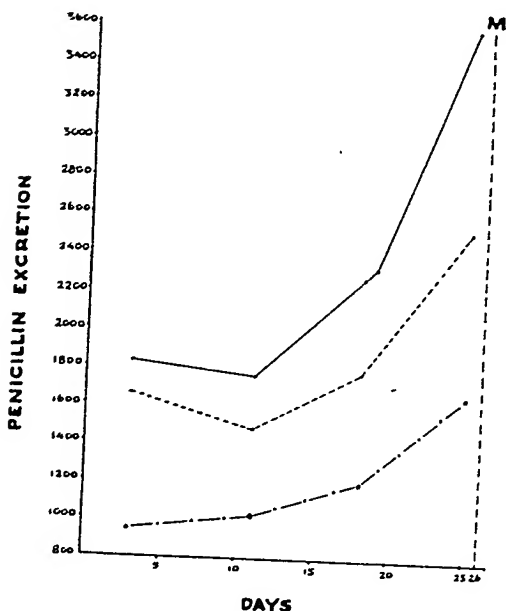


FIG. 1.

Urinary excretion of penicillin in Subject A at various intervals of the menstrual cycle. M signifies the onset of menstruation lasting three days.

— Sodium bicarbonate
 - - - Sodium citrate
 . . . Antacid mixture

bicarbonate U.S.P., sodium citrate U.S.P. and an antacid powder containing sodium bicarbonate, colloidal kaolin, magnesium trisilicate and bismuth subcarbonate. Physiological saline served as control. Urine was collected in sterile glass-stoppered bottles one-half hour after penicillin administration and thereafter at hourly intervals for 6 hours. The volumes of urine were determined, and the samples were assayed, using the FDA cylinder-plate method of January 1945 (*Staphylococcus aureus* FDA No. 209). A standard penicillin assay curve was prepared daily for comparison with the unknowns.

When the frequency and duration of the menstrual periods were established for each subject, identical doses of penicillin plus antacid were given with reasonable spacing from

4 to 6 times during the cycle, excluding actual menstruation. The test was then interrupted for at least one month to avoid any interference with subsequent doses and possible tolerance. A different antacid was then employed in the following month's tests.

Results. Fig 1 is a representative example of the 3 cases studied. It shows penicillin (expressed in Oxford units) recovered from the urine in 6 hours with different buffers at various intervals of the menstrual cycle, plotted against the number of days elapsed since the last day of menstruation. The first one-half hour sample includes the urine already present in the bladder prior to the test. Each point of the graph represents the average of at least 2 determinations.

It is apparent from the data that the recovery of penicillin in the urine is increased just before menstruation. Oral administration of penicillin-saline solutions resulted in small penicillin recovery in the urine, confirming the statement that unbuffered penicillin is absorbed in the intestinal tract to a slight extent.³

Discussion. The increase in urinary penicillin excretion preceding menstruation may be attributed either to an increased absorption or decreased destruction of penicillin from the gastrointestinal tract, or to an increased secretion. The actual mechanism will be investigated further. The data here presented may aid in explaining the discrepant reports on the urinary penicillin recovery in women after the oral administration of buffered penicillin, carried out without regard to the menstrual cycle.^{1,2}

Summary. A marked increase in urinary excretion of orally ingested penicillin has been observed in women immediately preceding menstruation. The physiological mechanism of this phenomenon is under investigation.

³ Rammelkamp, C., and Keefer, C. J., *J. Clin. Invest.*, 1943, **22**, 425.

Action of Furacin in Delaying Growth of a Transplanted Fibrosarcoma in Mice.*

MORRIS N. GREEN[†] AND CHARLES E. FRIEDGOOD.[‡] (Introduced by Stuart Mudd.)

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Relatively little is known regarding the chemotherapy of tumors. Greenberg and Schulman¹ recently suggested that the search for new chemotherapeutic agents against neoplasms might be facilitated by using the "metabolite antagonism" approach. This concept has been extensively applied in research directed towards finding new antibacterial agents.

Furacin[§] (5-nitro-2-furaldehyde semicarbazone) inhibits the growth of a large number of gram positive and gram negative bacteria.² Green³ recently has shown that furacin inhibits bacterial enzymes involved in glucose and pyruvate metabolism. The following experiments were designed to test the effect of furacin on growth of a transplanted fibrosarcoma.

Materials and methods. Forty adult inbred C3H mice of the Andervont strain were used. They were all transplanted with an equal amount of mouse sarcoma S-13^{||} into the right axillary space.

* This work was supported by a grant from the Eaton Laboratories, Inc., Norwich, N.Y.

[†] Present address: School of Medicine, University of Missouri, Columbia, Mo.

[‡] Present address: Mt. Sinai Hospital, New York, N.Y.

¹ Greenberg, D. M., and Schulman, M. P., *Science*, 1947, **106**, 271.

[§] Furacin was supplied by Dr. L. Eugene Daily of the Eaton Laboratories.

² Dodd, M. C., *J. Pharm. Exp. Therap.*, 1946, **86**, 311; Shipley, E. R., and Dodd, M. C., *Surg., Gyn., Obst.*, 1947, **84**, 366.

³ Green, M. N., *Fed. Proc.*, 1948, **7**, 305; in press, *Arch. of Biochem.*

^{||} The original tumor was supplied by Dr. Margaret R. Lewis of the Wistar Institute. It gives 100% takes on transplantation in this strain of mice.

Furacin was prepared for injection by suspending finely powdered crystals in peanut oil, using a concentration of 200 mg per ml. The furacin was administered by subcutaneous injection in the dorsal region.

The animals were divided into 4 groups of 10 each and treated as follows:

Group I One week after transplantation, 0.1 ml of furacin suspension (containing 20 mg of furacin) was injected.

Group II Three days prior to transplantation, the animals were given 0.1 ml of the furacin suspension followed by another injection of the same dose a week after transplantation.

Group III This group were also injected with 0.1 ml of the furacin suspension 3 days prior to transplantation, again after one week and 2 weeks after transplantation.

Group IV This was the control group. The tumor was transplanted as in the other groups but no furacin was given.

All the animals were housed in wire cages and were fed Purina Fox Chow stock diet. The tumors were palpated periodically and the progress of growth recorded. At the time of death the animals were autopsied and sections of the tumor, lung, kidney and adrenal glands were prepared for microscopic examination.

Results. Table I shows the effect of furacin in prolonging the life span of the tumor-bearing mice. It will be noted in Table I that there was less than one chance in a thousand that the differences in the average survival of the furacin-treated and control groups could be due to chance alone. Although furacin exhibited a definite inhibitory effect on the growth of the tumor, eventually all the animals died as a result of the malignancy. The most effective retardation of growth was obtained with three doses of furacin (Group III). Under these conditions, the

TABLE I.
Survival Time in Days of Mice After Implantation of S-13 Tumor and Treatment with Furacin.

Group	Treatment*	Avg survival (M)	Range	Stand. Dev. (σ)	tt	P‡
I	20 mg furacin inj.	29.2	22-34	3.55	5.76	>0.001
II	40 " "	34.2	30-42	3.60	8.93	>0.001
III	60 " "	37.4	30-47	4.68	9.27	>0.001
IV	Control	20.9	18-25	2.63	—	—

* For details of treatment see under description of methods in text. Each group contained ten mice.

$$t = \frac{M_1 - M_2}{\sqrt{\frac{\sigma_1^2}{9} + \frac{\sigma_2^2}{9}}}$$

‡ A value of $t = 4.781$ gives a chance variation (P) of 0.001.

tumor transplant was not palpable for 2 weeks, while in the control group, the tumor was palpable at the end of 5 days following transplantation. There was no evidence of metastasis in any of the animals; the fibrosarcoma growing locally was well encapsulated. In the treated animals, there was microscopic evidence of cellular degeneration with pyknotic nuclei and decreased mitotic activity of the tumors in the treated mice as distinguished from those in the untreated controls. The furacin was largely absorbed because only negligible amounts of the drug were observed at autopsy at the site of injection.

It is of interest to note that adrenal enlargement was observed in the furacin treated animals. Histologically the fascicular zone of the cortex appeared hypertrophied. The effect of furacin on the adrenal cortex will be reported in a subsequent paper. Using furacin in the doses described above, there was no evidence of parenchymal damage to the liver, kidney or lungs.

Discussion. These experiments indicate that furacin may inhibit the growth of neo-

plastic tissue. The histological findings in the treated tumors indicate that furacin has a selective effect on the tumor tissue. Experiments are being continued to determine which factors explain the action of furacin in inhibiting tumor growth.

Summary. Furacin inhibits the growth of mouse sarcoma S-13 in C3H mice. The average survival time of the control mice after implantation of the tumor was 21 days. The average survival time of the furacin-treated mice varied from 29 to 37 days, depending on the amount of the furacin given. Histological examination of the treated tumors reveal some cellular degeneration and a lessened mitotic activity. Hypertrophy of the adrenal cortex and symptoms of B complex vitamin deficiency were observed in the furacin-treated animals.

The authors wish to thank Dr. Margaret Reed Lewis of the Wistar Institute for her generous advice and interest in this problem as well as for cooperation in supplying the tumors and animals used in these experiments. We are also indebted to Dr. Stuart Mudd for his interest and support.

Effect of "Neurotripsy" on the Partially Denervated Muscle of the Dog.*

J. N. FREDERICK AND A. J. KOSMAN. (Introduced by J. S. Gray.)

From the Department of Physiology, Northwestern University Medical School, Chicago, Ill.

Regenerating nerve fibers may produce an increased number of axone branches. Billig, van Harreveld, and Wiersma¹ have applied this principle in their treatment of paretic muscles by a procedure which they have called "neurotripsy." This technic consists of either crushing the nerve as it enters the paretic muscle—surgical "neurotripsy"—or of a forceful massage of the affected part which is directed at breaking nerve fibers within the muscle sheath—closed manual "neurotripsy." Upon regeneration of the traumatized or broken nerve fibers, it is assumed that an increased number of axone branches may innervate a greater number of motor end plates. According to these authors, "neurotripsy," performed on muscles paralyzed by anterior poliomyelitis, has resulted in an increase in strength of approximately one half of a grade rated according to the Lovett System. Maximum effect was observed after about one year, although a considerable proportion of the total improvement was evident at 3 months. Muscles of low rating seemed to respond better than those of higher ratings. Increases in action potentials were parallel to increases in strength.

Prior to the report by these authors, Hines, Wehrmacher, and Thomson² had found that crushing the tibial nerve to the partially denervated gastrocnemius muscle of the rat resulted in a decrease in strength and weight of the muscle. Hodes³ observed in patients with anterior poliomyelitis that there was a decrease in the action potentials of paretic muscles during the first four months following closed

manual "neurotripsy." From 4 to 8 months after the operation the action potentials were slightly greater than those of controls and at more than 8 months there was an average increase in action potentials of 22%. Muscles which showed no electrical activity before the operation likewise showed no electrical activity after the operation.

This article is a preliminary report on studies which were undertaken to determine the effect of closed manual "neurotripsy" on the partially denervated anterior tibial muscle of the dog. The plan was to produce a partial, pure motor denervation of the anterior tibial bilaterally, to subject one anterior tibial to the operation, and to use the other as the control. For this experiment to be valid the following conditions would have to be fulfilled:

1. The strength and weight of the two normal anterior tibial muscles must be closely correlated.
2. The contribution of the ventral root of each spinal nerve supplying the anterior tibial must be determined.
3. The contribution of the ventral root of each spinal nerve must be the same for both left and right anterior tibial muscles.
4. Following bilateral section of identical ventral roots, the rate of atrophy and strength loss of the two muscles must be closely correlated.

Method. The partial denervation was accomplished by sectioning the ventral root of L₆ bilaterally. Closed manual "neurotripsy"† was performed on one anterior tibial muscle three months later and the animal sacrificed 5 months after the initial lesion. Autopsy was performed to check for any nerve regen-

* This work was aided by a grant from the National Foundation for Infantile Paralysis, Inc.

¹ Billig, H. E., van Harreveld, A., and Wiersma, C. A. G., *J. Neuropath. and Exp. Neur.*, 1946, 5, 1.

² Hines, H. M., Wehrmacher, W. H., and Thomson, J. D., *Am. J. Physiol.*, 1945, 145, 48.

³ Hodes, R., *Fed. Proc.*, 1948, 7, 56.

† We wish to acknowledge the valuable instruction of Dr. R. T. McElvenny of the Department of Bone and Joint Surgery in the performance of this operative technic.

TABLE I.

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IV	Control	20.9	18-25	2.63	—	—

* For details of treatment see under description of methods in text. Each group contained ten mice.

$$tt = \frac{M_1 - M_2}{\sqrt{\frac{\sigma_1^2}{9} + \frac{\sigma_2^2}{9}}}$$

† A value of $t = 4.781$ gives a chance variation (P) of 0.001.

tumor transplant was not palpable for 2 weeks, while in the control group, the tumor was palpable at the end of 5 days following transplantation. There was no evidence of metastasis in any of the animals; the fibrosarcoma growing locally was well encapsulated. In the treated animals, there was microscopic evidence of cellular degeneration with pyknotic nuclei and decreased mitotic activity of the tumors in the treated mice as distinguished from those in the untreated controls. The furacin was largely absorbed because only negligible amounts of the drug were observed at autopsy at the site of injection.

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The authors wish to thank Dr. Margaret Reed Lewis of the Wistar Institute for her generous advice and interest in this problem as well as for cooperation in supplying the tumors and animals used in these experiments. We are also indebted to Dr. Stuart Mudd for his interest and support.

TABLE IV.
Average Strength and Weight in Grams of Anterior Tibial Muscles After Neurotripsy on One Side.

	2 mos. after neurotripsy; 5 animals			
	Treated	Untreated	t ratio	Corr. coef.
Strength direct stim.	1980	2140	.68	.819
Strength sciatic stim.	1700	1860	.88	.869
Weight	5.48	5.74	.88	.813

"neurotripsy" in the dog should be long enough to bring out any improvement resulting from the treatment. Our failure to demonstrate any such improvement in weight and strength of the treated muscles would indicate that in the dog this procedure does not increase the residual innervation of the partially denervated muscle. However, additional experimental animals with a greater degree of denervation are being run for a longer period of time following the "neurotripsy" procedure.

The results indicate that stimulation of the spinal nerves exclusive of L₆ yields approximately 1000 g of tension for each muscle. However, three months after bilateral resection of L₆, each muscle showed an average strength of approximately 1950 g and 5

months after bilateral resection a strength of approximately 1900 g. This may indicate that following partial denervation there may be an increase in the residual innervation as suggested by some investigators^{2,4,5} or hypertrophy of the remaining innervated muscle fibers. This phenomenon is being investigated further.

Summary. Treatment of the partially denervated anterior tibial muscle of the dog with closed manual "neurotripsy" produces no significant increases in wet weight and muscle strength 2 months after treatment.

⁴ Weiss, P., and Edds, Jr., *Am. J. Physiol.*, 1946, **145**, 587.

⁵ Van Harreveld, A., *Am. J. Physiol.*, 1945, **144**, 477.

16803 P

Isolation of Brucella from Apparently Healthy Individuals.

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In recent years the isolation of brucella organisms from cases of Hodgkin's disease¹ has caused considerable discussion. The confirmation of this finding in our laboratory and the statements in the literature that these organisms could be isolated from patients with various infections² led us to seek an explanation.

It seemed to us that the most likely ex-

planation was that infection with brucella was widespread, at least in certain regions, and that the organism persisted in people furnishing favorable foci. In order to test this hypothesis it was decided to explore the possibility that tissues affording good conditions for growth of brucella organisms might actually harbor them. Since brucella are known

¹ Parsons, P. B., and Poston, M. A., *South. Med. J.*, 1939, **32**, 7; Forbus, W. D., and Gunter, J. N., *South. Med. J.*, 1941, **34**, 376.

² Mettler, S. R., and Kerr, W. J., *Arch. Int. Med.*, 1934, **54**, 702; Chaiklin, N. W., and Schwinner, David, *Rev. Gastroenterol.*, 1943; **10**, 130; Amoss, H. L., *Internat. Clin.*, 1931, **4**, 93.

TABLE I.
Mean Weight and Total Strength in Grams of Anterior Tibial Muscles.

	No. animals	Left	Right	t ratio	Correlation coef.
Total strength	31	3870	4020	1.18	.82
Weight	17	7.24	7.23	.24	.98

TABLE II.
Average Strength in Grams Developed by Anterior Tibial Muscles by Stimulation of Ventral Roots of Spinal Nerves.

No. animals	Nerve stim.	Left	Right	t ratio	Correlation coef.
21	L ₄	108	100	.48	.40
21	L ₅	504	509	.05	.83
20	L ₆	2810	3015	.38	.80
20	L ₇	342	323	.20	.23

TABLE III.
Average Strength and Weight in Grams of Anterior Tibial Muscles Following Bilateral Resection of Ventral Root L₆

	3 mos. after oper.; 6 animals				5 mos. after oper.; 5 animals			
	Left	Right	t ratio	Corr. coef.	Left	Right	t ratio	Corr. coef.
Strength direct stim.	1917	2000	0.58	.989	1800	1660	.78	.972
Strength sciatic stim.	1683	1883	1.45	.990	1540	1400	.55	.948
Weight	5.56	5.23	1.41	.979	5.14	4.92	.49	.915

eration and to verify the resected root. All animals showing any signs of regeneration were discarded. The contribution of the ventral roots supplying the anterior tibial muscle was determined in a group of normal dogs by recording the maximal tension developed by the muscle upon electrical stimulation of the individual ventral roots (L₄ to L₇ inclusive). Tension development was measured by a torsion myograph of the Blix type.

The control animals were subjected to a bilateral section of ventral root L₆, and with no further treatment they were sacrificed three and five months following the production of the original lesions. Wet weights and tension development upon maximal direct and indirect stimulation of the muscles were determined. Statistical analysis of the experimental data was carried out by the method of paired comparisons.

Results. Our experimental findings may be summarized as follows:

1. There was no significant difference between mean strengths and weights of the two normal anterior tibial muscles, and a high de-

gree of correlation between the muscles of the same animal. (Table I).

2. L₆ supplied approximately 75% of the motor innervation to the anterior tibial. L₄, L₅, and L₇ supplied the remaining motor fibers (Table II).

3. In the individual animals the contribution of each L₅ and L₆ was practically the same for left and right muscles as indicated by the correlation coefficient (Table II).

4. At 3 months and at 5 months after bilateral resection of the ventral root of L₆ the loss of strength and degree of atrophy of the left and right anterior tibials was the same (Table III).

5. Two months after "neurotripsy," there was no significant difference in either weight or strength between the treated and untreated muscle (Table IV).

Discussion. Billig, van Harreveld, and Wiersma found that a considerable proportion of the improvement in their patients was evident at 3 months after "neurotripsy;" likewise, a better response was obtained on poorer muscles. The two-month interval following

16804

Effect of the Amino Acid Hexahomoserine on Growth and Hematopoiesis in Swine.

E. T. MERTZ, W. M. BEESON, R. H. WALTZ, JR., AND R. GAUDRY.
(Introduced by W. A. Hiestand.)

From the Departments of Agricultural Chemistry and Animal Husbandry,* Purdue University, Lafayette, Ind., and the Department of Biochemistry, Medical School, Laval University, Quebec, Canada.

The synthesis of the amino acid, α -amino- ϵ -hydroxycaproic acid, was accomplished recently by one of the authors,¹ and the name hexahomoserine, was proposed for the new compound. It was shown that hexahomoserine could not replace lysine in the diet of rats, and that it was probably toxic.² Further studies³ showed that the incorporation of hexahomoserine in the diet of the rat caused a drop in the hemoglobin level, red cell count, and red cell volume of the blood. Evidence was cited³ indicating that hexahomoserine may be identical with the anemia-producing factor of deaminized casein.⁴

Inasmuch as feeding experiments with hexahomoserine have been limited to the rat, it appeared desirable to determine whether this compound would produce anemia in an unrelated species. The data presented below show that young pigs develop anemia and in addition, stop growing, when small amounts of hexahomoserine are added to an otherwise normal diet.

Experimental. Two male and 2 female purebred Duroc weanling pigs, whose weights ranged from 53-60 lb, were paired for the feeding experiments. The 4 pigs were kept in individual pens and were fed twice daily with amounts to satisfy the individual appetite of each animal. All animals received, throughout the course of the experiment, a

ration consisting of ground corn 58.75%, ground wheat 20%, Purdue supplement V 20% (this supplement consists of meat and bone scraps 20%, fish meal 20%, soybean oil meal 40%, cottonseed meal 10%, and alfalfa leaf meal 10%), mineral mixture 1% (this consists of equal parts of limestone, steamed bone meal, and salt), and concentrated cod liver oil (NOPCO XX) 0.25%.

For a period of 27 days, male 72 and female 70 each received, in addition to the above diet, 3 g of DL-hexahomoserine daily. The hexahomoserine was mixed with the first half of the daily ration. The other pigs, male 73 and female 71, received only the basal ration.

A record was kept of daily food consumption and weekly weight gains. Twice a week red blood cell,⁵ white blood cell,⁵ platelet,[†] and reticulocyte⁵ counts were made, and red blood cell diameters⁵ were measured, in blood samples taken from a lancet wound in the ear tip. Once a week, red blood cell, hemoglobin,⁶ cell volume,⁷ icteric index,⁵ prothrombin,⁸ non-protein nitrogen,⁹ total plasma pro-

* Journal paper No. 347, Purdue University Agricultural Experiment Station.

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⁵ Todd, J. C., and Sanford, A. H., *Clinical Diagnosis by Laboratory Methods*, pp. 238, 249, 294, and 386, W. B. Saunders Co., Philadelphia, 1936.

[†] Determined by measuring the ratio of platelets to red blood cells in blood diluted with 12% MgSO₄ and stained with Wright's stain.

⁶ Evelyn, K. A., *J. Biol. Chem.*, 1936, 115, 63.

⁷ Wintrobe, M. M., *J. Lab. Clin. Med.*, 1929, 15, 287.

⁸ Ziffren, S. E., Owen, C. A., Hoffman, G. R., and Smith, H. P., *Proc. Soc. Exp. Biol. and Med.*, 1939, 40, 595.

⁹ Hawk, P. B., and Bergeim, O., *Practical Physiological Chemistry*, pp. 420 and 453, P. Blakiston Co., Philadelphia, 1937.

to multiply in macrophages and fibroblasts,³ it was thought that cultures of enlarged prostates and fibrosed fallopian tubes might reveal the presence of these organisms.

At present 34 prostates have been cultured. From these cultures *Brucella abortus* has been isolated in 2 instances and *Brucella melitensis* in a third. Forty-three fallopian tubes have been similarly studied. From one of these *Brucella melitensis* has been isolated. The cultures of the prostates and tubes were made by obtaining from the surgeon, in a sterile container, portions of the prostate which were removed through the urethra. The specimens were immediately macerated, extracted, and the tissue extract incubated in Bacto-tryptose Broth at 37.5°C in 10% CO₂ for 10 days. After 10 days the broth specimens were inoculated on Bacto-Tryptose Agar plates, and these were similarly incubated for 10 days. Unless positive specimens were obtained sooner, this procedure was repeated twice before discarding the specimens as negative. Identification of the organisms was made by 1) the morphology of the colony on tryptose agar, 2) the morphology and staining reactions of the individual organisms, 3) sugar reactions, and 4) agglutination with specific antiserum. Two of the positive specimens were sent to another laboratory where their identification was confirmed.*

Microscopic examination of all the prostates

³ Goodpasture, E. D., and Anderson, K., *Am. J. Path.*, 1937, 13, 149; Castaneda, M. R., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 298.

* Two of the positive cultures were sent to the laboratory of Dr. W. W. Spink of the University of Minnesota School of Medicine where their identity was confirmed.

and all the fallopian tubes was made. Practically all of them showed evidence of chronic infection. Sections from 2 of the prostates and the fallopian tube had a perivascular reaction which was not present in the others. This reaction consisted in a perivascular cuffing with mononuclear cells, many of which were eosinophilic. The third prostate could not be evaluated microscopically, because of extensive involvement with cancer.

Although none of the patients from whom brucella were obtained had any unusual symptoms prior to operation, a review of their histories revealed certain significant facts. In all 4 cases there was a history of country life and contact with cows and other farm animals. In 1 instance the patient had been in contact with an animal infected with brucellosis. In all cases there was a history of the consumption of raw milk. All of the patients had a clinical record of illnesses compatible with brucellosis. None of these illnesses, however, had occurred in recent months. The serum of all 4 patients lacked agglutinins for brucella, while their skin gave strongly positive reactions with brucella antigen. The post operative course of these patients was characterized by persistent low grade elevation of their temperature. It was impossible, however, in any of the cases to be certain that this fever was due to the brucella since in every instance there were other complications which might account for it.

We believe that this study, together with those of others, indicates that brucella is endemic, at least in certain parts of the world, and that the organisms may be carried by a number of apparently healthy individuals.

Effect of the Amino Acid Hexahomoserine on Growth and Hematopoiesis in Swine.

E. T. MERTZ, W. M. BEESON, R. H. WALTZ, JR., AND R. GAUDRY.
(Introduced by W. A. Hiestand.)

From the Departments of Agricultural Chemistry and Animal Husbandry,* Purdue University, Lafayette, Ind., and the Department of Biochemistry, Medical School, Laval University, Quebec, Canada.

The synthesis of the amino acid, α -amino- ϵ -hydroxycaproic acid, was accomplished recently by one of the authors,¹ and the name hexahomoserine, was proposed for the new compound. It was shown that hexahomoserine could not replace lysine in the diet of rats, and that it was probably toxic.² Further studies³ showed that the incorporation of hexahomoserine in the diet of the rat caused a drop in the hemoglobin level, red cell count, and red cell volume of the blood. Evidence was cited³ indicating that hexahomoserine may be identical with the anemia-producing factor of deaminized casein.⁴

Inasmuch as feeding experiments with hexahomoserine have been limited to the rat, it appeared desirable to determine whether this compound would produce anemia in an unrelated species. The data presented below show that young pigs develop anemia and in addition, stop growing, when small amounts of hexahomoserine are added to an otherwise normal diet.

Experimental. Two male and 2 female purebred Duroc weanling pigs, whose weights ranged from 53-60 lb, were paired for the feeding experiments. The 4 pigs were kept in individual pens and were fed twice daily with amounts to satisfy the individual appetite of each animal. All animals received, throughout the course of the experiment, a

ration consisting of ground corn 58.75%, ground wheat 20%, Purdue supplement V 20% (this supplement consists of meat and bone scraps 20%, fish meal 20%, soybean oil meal 40%, cottonseed meal 10%, and alfalfa leaf meal 10%), mineral mixture 1% (this consists of equal parts of limestone, steamed bone meal, and salt), and concentrated cod liver oil (NOPCO XX) 0.25%.

For a period of 27 days, male 72 and female 70 each received, in addition to the above diet, 3 g of DL-hexahomoserine daily. The hexahomoserine was mixed with the first half of the daily ration. The other pigs, male 73 and female 71, received only the basal ration.

A record was kept of daily food consumption and weekly weight gains. Twice a week red blood cell,⁵ white blood cell,⁵ platelet,[†] and reticulocyte⁷ counts were made, and red blood cell diameters⁵ were measured, in blood samples taken from a lancet wound in the ear tip. Once a week, red blood cell, hemoglobin,⁶ cell volume,⁷ icteric index,⁵ prothrombin,⁸ non-protein nitrogen,⁹ total plasma pro-

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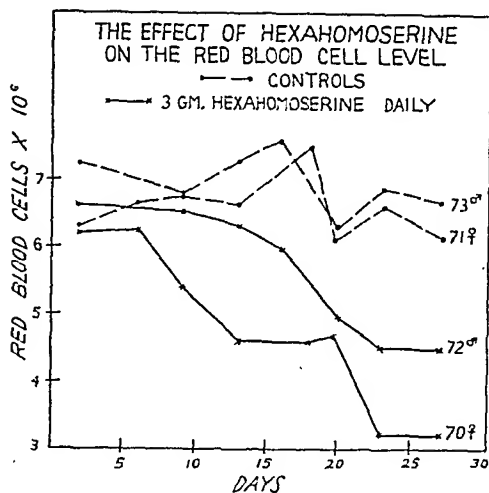


FIG. 1.

tein,⁹ albumin,⁹ globulin,⁹ and fibrinogen⁹ determinations were made on blood samples drawn from the anterior vena cava.

Results. The effect of hexahomoserine on the red blood cell level of the swine is shown in Fig. 1. The red blood cell level of pig 70 began to fall about 9 days after hexahomoserine had been added to the diet. By the 27th day, the red cell count had dropped to 3.2 million. At this point, the feeding of hexahomoserine was discontinued. One week after hexahomoserine was removed from the diet, the red cell count of this pig was still 3.2 million. The following day the pig died. Autopsy[†] showed marked enlargement of the spleen, lesions of swine pox, and a myocarditis characterized by accumulations of mononuclear cells. Microscopic examination of the bone marrow revealed no abnormal increase or reduction in the content of immature red blood cells. The bone marrow appeared to be functioning normally in the anemic animal.

The curve in Fig. 1 shows that pig 72 did not have a lowered erythrocyte count until about the 20th day after hexahomoserine had been added to the diet. On the 27th day, the last day on which the compound was fed, the erythrocyte count was 4.4 million, as com-

pared with 6.6 million for the control pig 73. One week after the feeding of hexahomoserine had been discontinued, the erythrocyte count of pig 72 had risen to 5.1 million, and the hemoglobin level and cell volume were back to normal (15.3 g of Hb and 35.7 ml of total cells per 100 ml of blood compared with 15.2 g of Hb and 36.2 ml of total cells for pig 73). Ten days after the removal of hexahomoserine from the diet, the erythrocyte count of pig 72 had risen to 6.1 million. The animal was maintained on the basal ration for a total of 5 weeks after the hexahomoserine was dropped from the diet, and was then autopsied. Macroscopic and microscopic examination of the various tissues, including the bone marrow, showed no abnormalities; the ingestion of hexahomoserine had apparently caused no permanent damage.

The hemoglobin and cell volume values obtained on the blood of the four pigs are summarized in Table I. The blood of pig 70 shows a marked drop and the blood of pig 72 a slight drop in hemoglobin and cell volume.

The white blood cell, platelet and reticulocyte counts, the red blood cell diameter, and the icteric index, prothrombin, non-protein nitrogen, total plasma protein, albumin, globulin and fibrinogen values obtained on the blood of the two pigs receiving hexahomoserine showed no significant deviations from the values obtained simultaneously on the 2 normal control pigs. The experimental data are not reproduced here. It should be pointed out that the level of hexahomoserine fed in these studies may have been too low to produce changes in any blood constituent except the red blood cell.

In addition to depressing the level of red blood cells, hexahomoserine affected the growth rate of the pigs (Fig. 2). The compound appeared to have a delayed action on growth, for the two animals consumed normal quantities of feed and gained weight at a normal rate for approximately one week after the compound was added to the diet. After this initial period, however, both animals consumed less feed and stopped growing. During the last 18 days of the experiment, the animals

[†] We are indebted to Dr. L. P. Doyle, Department of Veterinary Science, Purdue University, for performing the autopsies on pigs 70 and 72.

TABLE I.
Effect of Hexahomoserine on Hemoglobin Level and Blood Cell Volume of Swine.

No. of days on diet	Hemoglobin (g Hb/100 ml blood)				Cell volume (ml cells/100 ml blood)			
	70 ♀ *	71 ♀ †	72 ♂ *	73 ♂ †	70 ♀	71 ♀	72 ♂	73 ♂
0	12.8	15.1	14.9	12.4	31.0	36.5	36.0	31.0
6	14.4	14.3	—	—	29.5	33.0	—	—
12	9.9	14.8	13.2	15.2	29.7	35.9	32.8	39.4
18	10.3	15.0	12.0	15.6	25.0	34.6	29.6	35.6
27	6.4	15.0	12.5	14.7	22.5	39.5	32.0	39.0

* Animal received 3 g of hexahomoserine daily.

† Control animal. Received no hexahomoserine.

receiving hexahomoserine each consumed an average of 2.2 lb of feed per day, and gained little or no weight; the control animals each consumed an average of 3.5 lb of feed per day and gained weight in a normal manner.

Removing hexahomoserine from the diet of pigs 70 and 72 did not cause an immediate resumption of growth. Pig 70, when returned to the basal ration, lost 6 pounds during the first week and succumbed on the eighth day. Pig 72 recovered from the anemia in about 10 days but did not show normal weight gains for one month.

Discussion. The data presented above indicate that no major constituent of the blood except the red blood cell is affected when 3 g of DL-hexahomoserine are fed daily for 27 days to young (50-60 lb) pigs. If the decrease in circulating red cells in the blood of these pigs were due to a destruction of the red cells by hexahomoserine, one would expect an increase in the icteric index of the blood. No increase was observed. It is possible that hexahomoserine slows down or prevents the release of mature red cells to the blood stream. The finding of a normal supply of immature cells in the bone marrow of the anemic pig 70, and the presence of a normal icteric index and reticulocyte count in both of the anemic animals would support this hypothesis.

The inhibition of growth by hexahomoserine may be due to the development of a lysine deficiency in the animals. No data are available on the lysine requirements of swine, but lysine has been found essential for growth in all species tested to date. Hexahomoserine is closely related structurally to lysine, and may be a metabolic antagonist of the latter. Further studies on the mechanism of the

action of hexahomoserine are in progress.*

Summary. Two Duroc pigs were fed hexahomoserine (α -amino, ϵ -hydroxy caproic acid) for a period of 27 days. A marked lowering of the red blood cell count was observed in both animals. No major constituent of the blood, except the red blood cell, was affected. In addition to developing anemia, the pigs made no significant weight gains after one week on the diet containing hexahomoserine, and it is concluded that the amino acid inhibits growth.

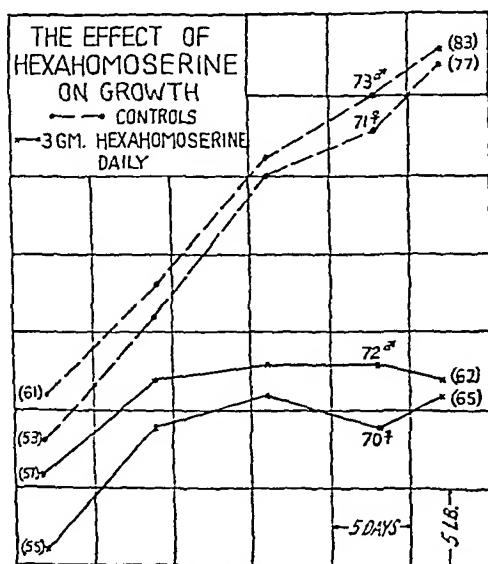


FIG. 2.

The numbers in parentheses denote the initial and final weights of the pigs.

* Since this paper was written, it has been shown conclusively that lysine is essential for the growth of pigs (Mertz, E. T., Shelton, D. C., and Beeson, W. M., *J. Animal Sci.*, 1948, 7, 530).

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